

**Anneli Pauli**

**The role of *Acinetobacter* sp. in biological phosphorus removal from forest industry wastewaters**

Yhteenveto: *Acinetobacter* sp. metsäteollisuuden jätevesien biologisessa fosforinpoistossa



# 16

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## THE ROLE OF *ACINETOBACTER* SP. IN BIOLOGICAL PHOSPHORUS REMOVAL FROM FOREST INDUSTRY WASTEWATERS

Anneli Pauli

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Activated sludge treatment with enhanced biological phosphorus removal based on the ability of some bacteria to accumulate excess phosphorus was studied as an option for the treatment of forest industry wastewaters. Wastewater and sludge samples were obtained from the existing, conventional aerobic activated sludge treatment plants of two sulphate pulp mills and three paper mills during 1991 - 1992. *Acinetobacter*s isolated in sludge samples were considered as model organisms for polyphosphate accumulating bacteria. *Acinetobacter*s constituted usually less than 2 % of the cultivable aerobic heterotrophic bacterial population in the samples. These bacteria could, however, be easily enriched in sludge. Lack of suitable carbon sources, such as volatile fatty acids, is one important factor limiting the growth of *acinetobacter*s in the treatment plants of sulphate pulp mills. Wastewater from integrated producing mechanical pulp and paper may contain enough of volatile fatty acids. For generation of suitable carbon compounds and selection for *acinetobacter*s against other aerobic heterotrophs a fermentative anaerobic phase (*acinetobacter*s tolerate anaerobiosis) would be needed in the treatment process. Cations (K, Mg, Ca) essential for growth and polyphosphate metabolism of *acinetobacter*s were not found to be limiting in the wastewaters studied. The growth of *Acinetobacter* pure cultures was enhanced with a higher nitrogen to phosphorus ratio in the medium than is common in forest industry wastewaters. The phosphate uptake by the phosphorus-starved *Acinetobacter* biomass (overplus phenomenon) as well as uptake by growing *Acinetobacter* cultures (luxury uptake under favourable growth conditions) followed the Michaelis-Menten model. The kinetic parameters obtained for the *Acinetobacter* isolates varied. One isolate originating from phosphorus-poor influent wastewater was more efficient in phosphate uptake than isolates from enrichment cultures or sludge amended with phosphorus. In spite of their high phosphate uptake rate the *Acinetobacter* populations present in the aerobic treatment processes studied could remove only a few percent of phosphorus from wastewaters. Anaerobic phosphate release, which is a prerequisite for efficient phosphorus removal, varied significantly so that 0 - 70 % of the aerobically bound phosphate was released during 24 h anaerobiosis. Release was more dependent on pH and the phosphate concentration in the medium than on the redox potential. Since there may be significant differences in phosphate uptake and release kinetics of *acinetobacter*s, the number of *acinetobacter*s alone would not be a reliable parameter for the phosphorus removal potential of sludge. The question of the denitrification ability of *acinetobacter*s has been raised in the literature, when simultaneous phosphorus and nitrogen removal is aimed at. None of the 57 *Acinetobacter* isolates studied denitrified.

Keywords: *Acinetobacter*, polyphosphate, enhanced biological phosphorus removal, forest industry

# 1 INTRODUCTION

## 1.1 Nutrient load from forest industry in Finland

In many industrialized countries phosphorus removal from wastewater is required by the authorities. Some countries have also set limits for nitrogen discharges. The requirements for nutrient removal ("nutrient" here refers to the main macronutrients nitrogen and phosphorus) usually concern only municipal wastewaters. In Finland phosphorus is removed from municipal wastewaters by chemical precipitation at almost all treatment plants. Nitrogen is removed only to a minor extent.

During the last few years nutrient discharges from pulp and paper industry, as well as diffuse loading from agriculture, have received attention due to the increased eutrophication of inland and coastal waters. In forest industry wastewaters the concentration of phosphorus is relatively low, but due to the large amount of wastewaters, the total phosphorus load to the recipient may be high and increase eutrophication. Upper limits for phosphorus loads have already been set for some mills.

In forest industry wastewaters most of the nutrients originate from the raw material, wood. Furthermore, many chemicals used in pulp and paper production contain nitrogen and phosphorus. A description of the characteristics of forest industry wastewaters is given by Puhakka (1990).

There are approximately 50 pulp and paper mills in Finland (Junna and Ruonala 1991). In 1989 pulp and paper mills were responsible for most of the BOD<sub>7</sub> (biological oxygen demand) load, half of the phosphorus load and one fifth of the nitrogen load from point sources (Table 1).

During the last 20 years, the measures taken both in the production methods and in effluent treatment, as well as the closing down of the sulphite pulp mills, have led to significant decreases in the loads of suspended solids and organic matter (measured as BOD<sub>7</sub>) from the forest industry in Finland.

Phosphorus and nitrogen loads from forest industry, however, increased in the 1980s reaching a maximum in 1988 (Fig. 1). This increase was mainly due to the growth of production (Junna and Ruonala 1991, Wirkkala 1992). It was then that a preliminary decision to

Table 1. Loads of organic matter as biological oxygen demand (BOD<sub>7</sub>), total concentration of phosphorus (P<sub>tot</sub>) and total concentration of nitrogen (N<sub>tot</sub>) from point sources to aquatic recipients in Finland in 1989 (Enckell-Sarkola and Kosola 1991).

Source	Load from point sources (t a <sup>-1</sup> )		
	BOD <sub>7</sub>	P <sub>tot</sub>	N <sub>tot</sub>
Pulp and paper industry	112 074	744	4 404
Other industry	7 762	88	1 916
Fish farming	(23 000) <sup>a</sup>	250	1 740
Municipalities	11 000	440	14 500
All point sources	153 836	1 522	22 560
All industry	119 836	832	6 320

a) an estimate

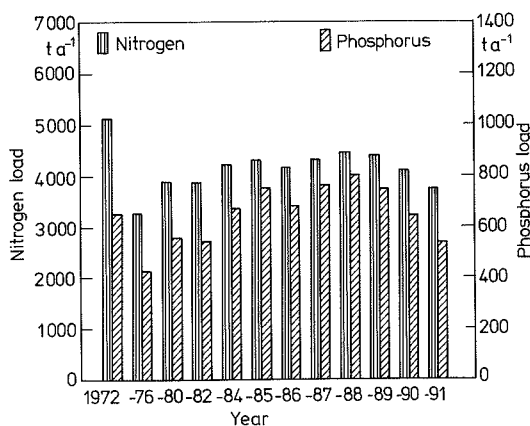


Fig. 1. Nitrogen and phosphorus loads to the aquatic recipients from pulp and paper industry in Finland in 1972 - 1991 (data by the National Board of Waters and the Environment).

launch the present study was made. Due to an improved control of activated sludge plants the phosphorus load started to decrease at the very end of the decade, reaching in 1991 the same level as at the end of the 1970s (statistics from the National Board of Waters and the Environment).

In accordance with the recommendations of the Baltic Marine Environment Protection Commission (the Helsinki Commission) and the Water Protection Programme to 1995, introduced by the Finnish Council of State (Ministry of the Environment 1988), the total phosphorus load from pulp and paper industry will be



reduced to  $1.5 \text{ t d}^{-1} \text{ P}$  by 1995 (about  $2.2 \text{ t d}^{-1} \text{ P}$  in 1988), calculated as the annual mean value. This goal was almost achieved already in 1991 (c.f. Fig. 1). However, loads from some pulp and paper mills may be high, and thus there is still a great need for more efficient phosphorus removal technologies (Hynninen 1993).

## 1.2 Treatment of forest industry wastewaters in Finland

In the 1970s almost all mills had mechanical wastewater treatment. The first biological wastewater treatment system, an aerated lagoon, was introduced in 1972. Because of the more stringent requirements for removal of organic matter in wastewaters, more efficient biological treatment methods were needed. The first activated sludge plant was started in 1984. At the end of 1992 there were 26 activated sludge plants and a few aerated lagoons and anaerobic processes in operation. More than half of the mills had biological wastewater treatment, and approximately 80 % of the wastewater was treated biologically (Table 2). By the year 1995 all major mills are likely to have some kind of biological treatment system (Junna and Ruonala 1991).

Forest industry wastewaters are nutrient deficient relative to carbon. To ensure good microbial growth in activated sludge (i.e. to maximize the degradation and removal of organic matter) nitrogen (usually as urea) and phosphorus (as phosphorus acid or in pulp mills also as lime mud from the recovery boiler) has been commonly added to the treatment process (Junna

and Rintala 1990). It has been found lately that there may be enough of phosphorus in pulp mill wastewaters (e.g. Lammi and Pakarinen 1993, Puustinen 1993), and nowadays phosphorus is not always added.

The first activated sludge treatment plants for forest industry wastewaters suffered from operational problems, and phosphorus was sometimes overdosed. According to a survey made in 1987 phosphorus concentration in the effluent was reduced by 20 to 40 % as compared with the influent wastewater from the mill. In some cases there was more phosphorus in the effluent than was discharged to the treatment plant from the mill. This was partly due to poor removal of suspended solids in the clarifier, but probably also to phosphorus additions (Junna and Rintala 1990).

Due to increasing experience and research on the activated sludge process carried out by the mills themselves, the three-year non-public MEBITE project (Hynninen 1993) and the three-year Environmental Research and Development Programme for the Finnish Forest Industry or the SYTYKE programme (Jørgensen and Pauli 1992, Lammi and Pakarinen 1993, Puustinen 1993, Ruonala, in press), the control of phosphorus levels in the process has been improved and loads decreased. At least in some cases phosphorus levels can be controlled simply by adjusting the phosphorus addition so that phosphate concentrations at the end of the aeration phase are only slightly above the analytical detection limit. Variations in the amount of organic matter and the phosphorus content of the sludge also affect the phosphorus dosage (Wirkkala 1992, Puustinen 1993, Saunamäki et al. 1993).

Table 2. Wastewater treatment plants of the Finnish pulp and paper mills in 1992 (statistics by the National Board of Waters and the Environment).

Type of the treatment plant	Number of plants	Percentage of wastewater treated
Activated sludge	26	69
Aerated lagoon	3	9
Anaerobic process	2	3
Chemical precipitation and flocculation	7	4
Mechanical treatment	8	15
All	46	100

## 1.3 Activated sludge process

Phosphorus removal from wastewater can be achieved through chemical precipitation and coagulation, through biological treatment or a combination of both. Different methods of phosphorus removal have been reviewed e.g. by Yeoman et al. (1988).

At present, the most common biological wastewater treatment method is the aerobic activated sludge process (Gray 1990). This process can be compared with a complex

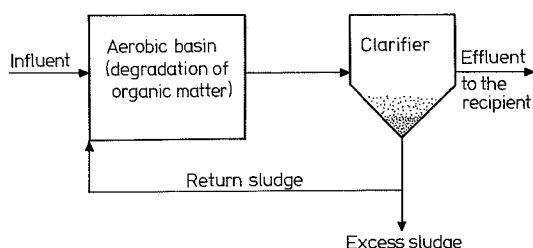


Fig. 2. The activated sludge process.

continuous culture with partial recycling of cells (Fig. 2). Microbes are retained in the system as “activated” sludge. The process consists of two phases, namely aeration and sludge settlement in a clarifier. Via microbial growth and respiration the organic matter of the influent is converted into new microbial biomass and inorganic compounds (e.g.  $\text{CO}_2$ ). Organic matter and nutrients are removed from the system by taking out part of the biomass. The rest of the biomass is returned to the aeration basin (so-called return sludge or recycled sludge).

In the conventional activated sludge treatment process not designed for enhanced biological phosphorus removal, bacteria usually take up phosphorus only in quantities satisfying their basic metabolic requirements.

Chemicals (aluminium, iron or calcium) can also be used in various stages of the activated sludge treatment process. Chemical precipitation is commonly included in the biological treatment of municipal wastewaters. Chemicals have also been tested in the treatment of sulphate pulping wastewater (Väänänen 1988). Chemical treatment increases the running costs and produces more sludge than purely biological treatment. The counterions of the added chemicals also contribute to the chemical pollution of the recipient.

Most activated sludge plants are unique, differing from each other with respect to the configuration and dimensions of the plant as well as with respect to the quality of the substrate, i.e. the influent.

Activated sludge had been used for purification of municipal wastewater (Arden and Lockett 1914) long before the first chemostat with the feedback of concentrated cells was introduced by Herbert (1961). This is a typical order of things in the development of biological wastewater treatment: the pressure to find immediate

solutions to environmental problems leads to full-scale applications and implementation of a treatment system before there is a proper scientific background for the biological mechanisms behind them (Wentzel et al. 1992).

The empirical approach to design activated sludge plants has been successful when the sole aim has been to remove suspended solids and oxygen consuming substances. Nowadays there are both environmental and economic pressures to develop the activated sludge process further e.g. for the following reasons:

- to achieve good phosphorus and nitrogen removal
- to purify complex, sometimes toxic industrial wastewaters
- to reduce sludge production
- to reduce or eliminate the use of chemicals
- to reduce energy consumption
- to reduce space taken by the plant
- to reduce operation costs in general
- to enhance the operational reliability of the purification process

Enhanced biological phosphorus removal based on the ability of some bacteria to accumulate excess phosphorus in their cells is an alternative to the traditional, aerobic activated sludge process. Phosphorus concentrations can be reduced to a very low level without chemicals, and the running costs of the treatment plants can be cut down through savings in the costs of chemicals and aeration energy (part of the treatment is anaerobic).

#### 1.4 Basis for enhanced biological phosphorus removal in the activated sludge process

Enhanced biological phosphorus removal in wastewater treatment has been studied for about 20 years in many countries (reviewed e.g. by Toerien et al. 1990), and there are successful full-scale applications in the treatment of municipal wastewaters (e.g. van Huyssteen et al. 1990, Rabinowitz et al. 1990, Randall et al. 1990, Goronszy 1992). Optimization and better control of the process would, however, require more information on the ecology and physiology of the polyphosphate accumulating bacteria (poly-P bacteria).

#### 1.4.1 Polyphosphate accumulating bacteria

According to the most commonly accepted theory, enhanced biological phosphorus removal is based on the enrichment of activated sludge with bacteria capable of accumulating orthophosphate ( $\text{PO}_4^{3-}$ ) in excess to the normal metabolic requirements of the cell. Orthophosphate is stored as polyphosphates.

The ability to take up excess amounts of phosphate is widespread among eukaryotic and prokaryotic microbes. Many bacteria isolated from activated sludge (laboratory or full-scale reactors), for instance *Acinetobacter* sp., *Aeromonas hydrophila*, *Pseudomonas* sp., *Moraxella* sp., *Enterobacter* sp., *Xanthobacter* sp., *Comamonas-Pseudomonas* group, *Paracoccus* sp., *Flavobacterium-Cytophaga* group, *Zoogloea ramigera*, some filamentous bacteria, coryneform bacteria and some unidentified Gram-positive bacteria, have been reported to accumulate polyphosphates (e.g. Roinestad and Yall 1970, Deinema et al. 1980, Lötter 1985, Lötter and Murphy 1985, Suresh et al. 1985, Hiraishi and Morishima 1990, Streichan et al. 1990, Nakamura et al. 1991, Okada et al. 1992). In addition, phototrophic *Rhodospseudomonas* strains (Hiraishi and Kitamura 1984), the yeast *Saccharomyces cerevisiae* (Urech et al. 1978), the fungus *Neurospora crassa* (Burns and Beever 1979, Cramer et al. 1980), and many planktonic algae and cyanobacteria (Kulaev and Vagabov 1983, Cembella et al. 1984, Bolier et al. 1992) are known to accumulate polyphosphates.

Excess phosphate uptake has been observed in various environments. In addition to wastewater treatment the ability of microbes to accumulate phosphate has received attention in eutrophication and sediment studies (Matinvesi and Heimonen-Tanski 1992, Davelaar 1993, Gächter and Meyer 1993, Sinke et al. 1993).

Most of the studies on poly-P bacteria in wastewater treatment have been made with sludge treating municipal wastewater. There are no previous studies on these bacteria in sludge treating forest industry wastewater.

Studies with municipal sludge have shown that bacterial populations in activated sludge are highly diverse, and the species composition changes continuously although the populations may be relatively stable at a generic level (Hantula et al. 1991). According to the biochemical models (chapter 1.4.2) the enrichment of poly-P

bacteria in sludge requires among other things both anaerobic and aerobic phases. It is likely that in permanently aerated treatment plants poly-P bacteria are not able to compete for substrates and are overgrown by more competitive aerobes (Marais et al. 1983). In systems with alternating anaerobic and aerobic conditions, denitrifiers may, if nitrate is present, compete with poly-P bacteria for fermentation products (Toerien et al. 1990).

Representatives of the genus *Acinetobacter* are usually considered to be the most significant and efficient phosphate accumulators in activated sludge treating municipal wastewater (e.g. Fuhs and Chen 1975, Deinema et al. 1980, Lötter and Murphy 1985, Lötter et al. 1986). It must, however, be borne in mind that most studies on the population structure of activated sludge have been based on the counts of colony forming units on agar plates (reviewed by Toerien et al. 1990), which sustain the growth of only part of the bacterial population. For instance Cloete and Steyn (1987) detected with plating techniques only 10 % of the microscopically visible sludge bacteria. Unz and Dondero (1970) were able to culture 14 % of the cells isolated from sludge by the micromanipulation technique.

For acinetobacters polyphosphate contents of up to 10 % (as P) of dry mass have been reported (e.g. Fuhs and Chen 1975, Deinema et al. 1980, Buchan, 1982, 1983, Lötter 1985, Lötter and Murphy 1985, Streichan et al. 1990, Okada et al. 1992).

However, the overall contribution of acinetobacters to biological phosphorus removal, their ability to survive in an aerobic-anaerobic system, as well as the theory that acinetobacters and other poly-P bacteria become enriched under alternating aerobic and anaerobic conditions have also been questioned (e.g. Brodisch and Joyner 1983, Cloete et al. 1985, Lötter et al. 1986, Stephenson 1987, Hiraishi et al. 1989a, Toerien et al. 1990).

In some studies with municipal sludge showing enhanced biological phosphorus removal acinetobacters have been found to account only for 1 to 10 % of the total bacterial population in laboratory and pilot scale activated sludge units (e.g. Brodisch and Joyner 1983, Hiraishi et al. 1989a). According to Cloete et al. (1985), Lötter et al. (1986) and Hiraishi et al. (1989a) enhanced biological phosphorus removal would not be due

to any major changes in the numbers of poly-P bacteria - i.e. enrichments - but instead, under appropriate conditions phosphate accumulation would be induced in the poly-P bacteria already present in the sludge. These authors do not, however, present any suggestions on the mechanism of induction of phosphate accumulation in bacteria.

It is obvious that also in treatment processes with enhanced biological phosphate removal the presence of acinetobacters and other poly-P bacteria depends on the process design and the composition of the influent. For instance in municipal sewage treatment plants with anaerobic and aerobic sequences *Acinetobacter* sp. belonged to the dominant (20–40 %) Gram-negative bacteria at low, but not, unexpectedly, at high concentrations of organic matter (Auling et al. 1991). In the study of Bonting et al. (1992b) *Acinetobacter* sp. constituted a major part of the bacterial population (in a phosphate-removing pilot plant (Renpho system), but only a minority of the population in a phosphate-removing laboratory-scale fill-and-draw activated sludge system.

Although acinetobacters are the most intensively studied and best known poly-P bacteria in the activated sludge, their taxonomy is obscure (Henriksen 1976, Bouvet and Grimont 1986, Beacham et al. 1990). Beacham et al. (1992) and Bosch and Cloete (1993) found no relationship between the taxonomic status of the *Acinetobacter* isolates and their ability to accumulate polyphosphate. There do not appear to be clear criteria for establishing distinct species and creating a widely accepted classification scheme. In a review of Juni (1984) all strains of *Acinetobacter* are classified as a single species, *A. calcoaceticus*. It is known, however, that the genus *Acinetobacter* is biochemically and genetically heterogenous. DNA hybridization studies have identified 12 distinct groups (genospecies). Species names have been proposed for six of these groups: *A. calcoaceticus*, *A. baumannii*, *A. haemolyticus*, *A. junii*, *A. johnsonii*, *A. lwoffii*. There are many different strains in each of these species (Bouvet and Grimont 1986).

Even closely related *Acinetobacter* strains may be different with respect to their phosphorus accumulation. Bark et al. (1992) found marked differences in the phosphorus accumulating ability of the *Acinetobacter* strains that were similar

with respect to their enzymes responsible for energy generation from polyphosphates (the polyphosphate:AMP phosphotransferase). The study of Bonting et al. (1992b) indicates that plasmids may have a role in polyphosphate accumulation. Bayly et al. (1991) found that within the same strain the deletion of some plasmid DNA can have an effect on phosphorus accumulation.

Some general characteristics of *Acinetobacter* sp. are summarized below. The information is based on a review of Juni (1984) if not otherwise stated.

*Acinetobacter* belong to the family *Neisseriaceae*. They are aerobic, Gram-negative, oxidase-negative and catalase-positive. *Acinetobacter* occur naturally in soil, water and sewage. It has been estimated that at least 0.001 % of the total heterotrophic aerobic bacterial population in soil and water are acinetobacters. *Acinetobacter* sp. is a non-motile rod (from 0.9 to 1.6  $\mu\text{m}$  in diameter and from 1.5 to 2.5  $\mu\text{m}$  in length). Rods become spherical in the stationary phase of growth. Cells can occur in pairs or chains. *Acinetobacter* possess all the enzymes of the tricarboxylic acid cycle (TCA-cycle) as well as those of the glyoxylate cycle (a modification of the TCA-cycle). Some strains can utilize glucose via the Entner-Doudoroff pathway under aerobic conditions. *Acinetobacter* grow well in all complex media, and most strains are able to grow in media containing a single carbon and energy source (e.g. ethanol, acetate, lactate, pyruvate, malate) and no growth factors. All strains grow between 20° C and 30° C, with most strains having optima between 33° C and 35° C. Optimum pH for growth is slightly below 7.

Competence for genetic transformation has been observed in a few strains. Lytic phages for acinetobacters have been isolated from sewage. Plasmids are found in many acinetobacters. Some plasmids can be transferred from *Pseudomonas* to *Acinetobacter*.

*Acinetobacter* have also other metabolic abilities than polyphosphate accumulation which would be useful in the treatment of forest industry wastewater. An *Acinetobacter* isolate has been reported to degrade lignin (including black liquor lignin) and other complex phenolic compounds in wood (Vasudevan and Mahadevan 1992).

Some acinetobacters can degrade recalcitrant compounds and hydrocarbons (Asperger and

Kleber 1991, Fewson 1991, Gutnick et al. 1991).

Acinetobacters are usually considered to be unable to denitrify, i.e. use nitrate as an electron acceptor and reduce it to nitrogen gas (Juni 1984). There are, however, reports on acinetobacters from sludge being able to denitrify (e.g. Lötter et al. 1986). The question of denitrification in acinetobacters is of practical importance for biological nutrient removal aiming at simultaneous phosphorus and nitrogen removal.

A consortium of bacteria may be important in enhanced phosphorus removal (Brodisch 1985, Ye et al. 1988). Brodich (1985) has reported that the presence of *Aeromonas punctata* increased phosphate uptake of *Acinetobacter calcoaceticus* by fermenting carbohydrates to volatile fatty acids, which are essential in polyphosphate accumulation (chapter 1.4.2).

Interactions between protozoa and polyphosphate bacteria have received little attention. Buchan (1983) observed in municipal sludge that amoebas contained bacterial cells with polyphosphate granules. It seemed as if the amoebas grazed only on these cells.

#### 1.4.2 Biochemical models

The theories and biochemical models presented to explain the biological basis for enhanced phosphorus removal are to some extent hypothetical and rely on knowledge of bioenergetics, membrane transport and biochemical pathways in bacterial cells in general and on experiments with pure cultures of poly-P bacteria and activated sludge. The models seem to be able to explain many, but not all of the observations in pilot or full-scale treatment plants.

Wentzel et al. (1991) have evaluated the latest biochemical models of biological phosphorus removal (Comeau et al. 1986, Wentzel et al. 1986, Mino et al. 1987), which are based on the earlier models. They concluded that there is a consensus regarding many of the biochemical pathways involved. The present state of knowledge on the biological basis of phosphate removal is summarized briefly below, indicating the main gaps. The subject has also been reviewed by e.g. Toerien et al. (1990) and Hilmer et al. (1991).

There are two main types of models, which are called the Comeau-Wentzel model (Fig. 3a – b)

and the Mino model (Fig. 3c – d). The Comeau-Wentzel model is based on the characteristics of *Acinetobacter* sp. as well as on the biochemical pathways operating generally in microorganisms. The model has been developed with acetate as a substrate, but it is also assumed to be valid for other similar simple carbon substrates. This model explains some observations with sludge as well (Bordacs and Chiesa 1989). The Mino model is based on laboratory experiments with sludge (i.e. mixed cultures) fed with acetate, propionate, glucose and peptone.

According to both models, a prerequisite for enrichment of poly-P bacteria, and hence for enhanced biological phosphorus removal, is recycling of sludge through anaerobic and aerobic phases. In anaerobic conditions, with no nitrate present, aerobic poly-P bacteria use their internally stored energy sources, i.e. polyphosphates, while facultatively anaerobic bacteria switch to fermentative metabolism (Nicholls and Osborn 1979).

According to this simple theory there is a clear difference between reactions in the aerobic and anaerobic phases. In the anaerobic phase carbon is taken up and polymerized, polyphosphates are degraded, and orthophosphate is released by the bacteria (or sludge). In the subsequent aerobic phase phosphate is taken up again and carbon polymers are degraded. The main factors regulating the biochemical pathways are ATP/ADP (adenosine triphosphate/adenosine diphosphate) and  $\text{NADH}_2/\text{NAD}$  (nicotinamide adenine dinucleotide, the reduced and oxidized forms, respectively) ratios (Wentzel et al. 1986). The synthesis and degradation of polyphosphates and organic carbon polymers are controlled by these ratios.

In wastewater treatment phosphorus is removed from the system after the aerobic phase by removing part of the bacterial mass rich in polyphosphates.

All observations do not comply with the simple model described above. Especially the function of the anaerobic zone is unclear (Toerien et al. 1990). One important function of anaerobiosis is likely to be the selection of acinetobacters and other poly-P bacteria against aerobic bacteria unable to form polyphosphates, but on the whole, the role of anaerobic conditions in phosphate release is not properly understood. For instance the results of Gerber et al. (1987a, b) indicate that phosphate

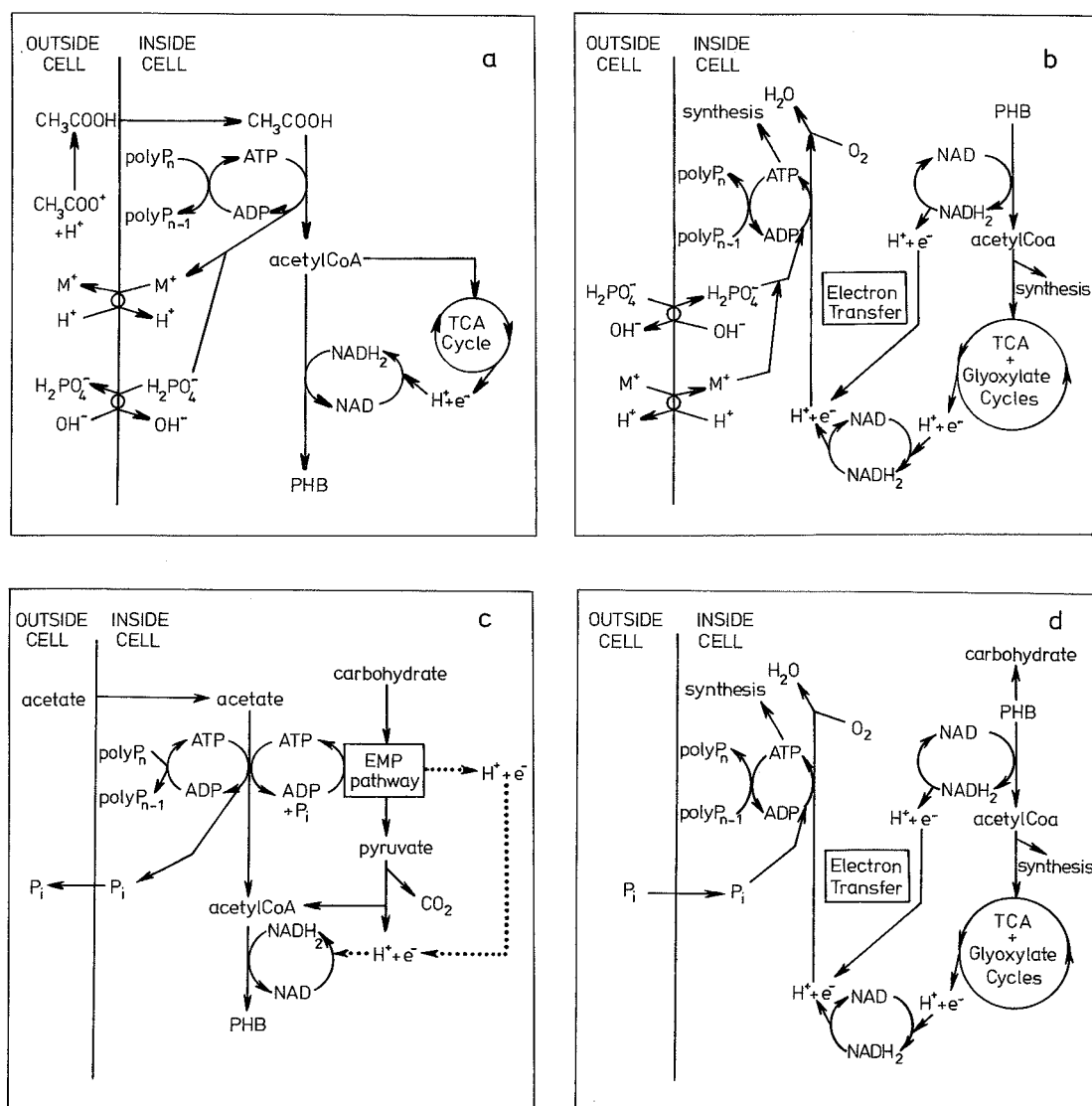


Fig. 3a – d. The biochemical pathways of polyphosphate metabolism according to the Comeau-Wentzel model under (a) anaerobic and (b) aerobic conditions, and according to the Mino model under (c) anaerobic and (d) aerobic conditions (by Wentzel et al. 1991).

release is controlled by the nature of the carbon substrate rather than the redox potential.

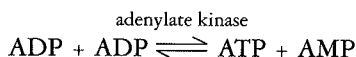
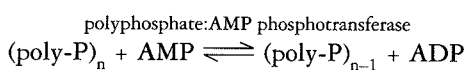
Polyphosphate accumulation may also take place under permanently aerobic conditions if suitable bacteria and appropriate carbon sources are present (Buchan 1982). *Acinetobacter* sp. is reported to constitute as much as 60 % of the cultivable bacterial population isolated from completely aerobic municipal activated sludge (Lötter and Murphy 1985, Lötter et al. 1986).

According to the biochemical models, under anaerobic conditions fermentative bacteria (non-poly-P bacteria) ferment organic compounds to volatile fatty acids and alcohols. Fermentation products (e.g. acetate) are transported to the cells of poly-P bacteria by diffusion if the concentrations of these substrates are high enough (Fig. 3a and 3c). In the cells fatty acids are reduced and stored as organic carbon polymers such as poly- $\beta$ -hydroxybutyrate (PHB) or poly- $\beta$ -

hydroxyvalerate (PHV), depending on the fatty acid. PHB and PHV are known by a generic name as poly- $\beta$ -alkanoates (Comeau et al. 1987a). It has been suggested that one of the roles of the anaerobic phase is to maximize the carbon storage of poly-P bacteria.

The main difference between the two models describing the anaerobic conditions is the source of reducing power ( $\text{NADH}_2$ ). According to the Comeau-Wentzel model the reducing equivalents ( $\text{NADH}_2$ ) are produced from acetate via the tricarboxylic acid cycle (TCA-cycle, Fig. 3a). In the Mino model  $\text{NADH}_2$  is formed through conversion of carbohydrate to pyruvate via the Embden-Mayerhof (EMP, Fig. 3c) or Entner-Doudoroff (EDP) pathways. In principle both models can be correct. *Acinetobacters* do not have the Embden-Mayerhof pathway (Juni 1984), whereas other poly-P bacteria probably possess this pathway.

Reduction of fatty acids to PHB and PHV requires energy, which is obtained from the breakdown of polyphosphates that were stored by the bacteria during the aerobic phase. The role of polyphosphates as an energy reserve under anaerobic conditions in *Acinetobacter* sp. has been shown by van Groenestijn et al. (1987) and Bonting et al. (1991). ATP is formed through the combined action of polyphosphate: AMP phosphotransferase and adenylate kinase. The energy generating reactions can be described by two equations:



The net result is degradation of polyphosphates and generation of ATP. Polyphosphate degradation and hydrolysis of ATP give rise to the release of inorganic phosphate, magnesium ( $\text{Mg}^{2+}$ ) and potassium ( $\text{K}^+$ ). These cations are expelled from the cell in order to maintain normal osmotic pressure. These reactions do not cause any net change of pH in the growth medium.

According to the models assimilation of volatile fatty acids, accumulation of PHB or PHV and degradation of polyphosphate would be linked to each other. However, simultaneous PHB or PHV accumulation and polyphosphate degradation has

not yet been proved experimentally to take place in the same organism. Hao and Chang (1987) did not observe acetate assimilation by an *Acinetobacter* isolate releasing phosphate. Appeldoorn et al. (1992b) did not find a relationship between PHB accumulation and phosphate release by sludge enriched with poly-P bacteria.

Under aerobic conditions there are only minor differences between the two models. In the aerobic phase PHB or PHV is broken down, phosphate is taken up, polymerized and stored as polyphosphates (Fig. 3b and 3d). Magnesium and potassium are also taken up by the cells. The pH value of the growth medium increases. PHB or PHV is used as a carbon and energy source for both bacterial growth and polyphosphate synthesis. According to the Mino model, part of the PHB is used for synthesis of carbohydrates. The mechanism of this conversion is not clear.

The Comeau-Wentzel model cannot explain the changes in the concentrations of intracellular and extracellular carbohydrates in anaerobic and aerobic conditions. In the pilot-scale studies performed by Abu-ghararah and Randall (1991) phosphate release and uptake could not be predicted on the basis of the Comeau-Wentzel model. It also remains to be proven whether *Acinetobacter* sp. is an acceptable model for poly-P bacteria.

The possible existence of denitrifying poly-P bacteria has not been taken into account in the models. Even if the denitrifying ability of acinetobacters is controversial (Juni 1984, Lötter 1985, Lötter et al. 1986), there might be other poly-P bacteria capable of denitrification (Carlsson et al. 1991a, b, Kuba et al. 1993). Bacteria produce less energy with nitrate than with oxygen as an electron acceptor. Thus, for a given amount of stored carbon polymer oxidized, less phosphate is taken up (Comeau et al. 1987a).

To conclude, according to both biochemical models the enrichment of aerobic poly-P bacteria (acinetobacters) during alternating aerobic and anaerobic conditions is due to their ability to take up and store fermentation products as organic carbon polymers (PHB or PHV) under anaerobic conditions, and to take up excess phosphate and store it as polyphosphates under aerobic conditions. Many other strictly aerobic bacteria do not have these properties. The energy for storing carbon is gained from the breakdown of polyphosphates. When moved from anaerobic to

aerobic conditions poly-P bacteria can utilize their carbon storage immediately as an energy and carbon source, and start multiplying without delay. In this way these bacteria may overgrow those obligate aerobes which are not able to store polyphosphates and form organic carbon polymers.

### 1.4.3 Polyphosphates

Phosphorus has many important functions in cells. It is present in many structural components of the cell, for instance in nucleic acids and phospholipids. Transformations of phosphorus compounds form the basis of cellular bioenergetics. The metabolism of polyphosphates has been reviewed by e.g. Harold (1966), Kulaev and Vagabov (1983) and Kulaev (1985).

Polyphosphates are linear, cyclic, cross-linked or branched polymers of orthophosphate with energy-rich phosphoanhydride bonds equivalent to the bonds in ADP and ATP. The number of phosphate residues in these compounds may vary from two to several thousand (Harold 1966, Kulaev and Vagabov 1983).

Polyphosphate anions are neutralized by cations (called counterions). Cations can also be important in the synthesis of polyphosphates. At least magnesium (Mg), calcium (Ca), potassium (K), manganese (Mn) and zinc (Zn) are known to be significant in polyphosphate metabolism (Buchan 1981, 1983, Kulaev and Vagabov 1983, Arvin and Kristensen 1985, Kulaev 1985, Comeau et al. 1987b, Davelaar 1989, Beacham et al. 1990). The presence and function of anions vary, depending on the microorganism and environment (van Groenestijn et al. 1988, Jensen and Corpe 1993).

In poly-P bacteria Mg is released and taken up simultaneously with phosphate. Mg was the most important counterion in *Acinetobacter* isolate 210A, but could be replaced by Ca (van Groenestijn et al. 1988). In this isolate the lack of K limited polyphosphate accumulation severely. Uptake and release of K did not seem to be directly linked with phosphate uptake and release, but rather with the energetic state of the cell (van Groenestijn et al. 1988). In activated sludge both Mg and K have been observed to be released simultaneously with phosphate (Mostert et al. 1988, Winter 1989). Nakamura et al.

(1991) found that K accelerated phosphate uptake in activated sludge, but Mg and Ca did not have any marked effect.

The exact physiological functions of polyphosphates in bacteria are not well known. According to Kulaev (1985) polyphosphates can be important in several cellular functions, e.g. (1) the accumulation of energy-rich ATP phosphorylic residues in an osmotically inert reserve material, (2) making cells more independent of environmental conditions through the accumulation of polyphosphate reserves (3) regulation of ATP and other nucleotide levels in cells, (4) fulfilling the function of ATP by participation in phosphorylation reactions, (5) linking cation metabolism with polyphosphate metabolism and (6) contributing to cellular homeostasis and osmotic regulation. Jensen and Corpe (1993) mention that polyphosphates may also neutralize the toxic effects of heavy metals.

According to van Groenestijn (1988) the most important role of polyphosphates for poly-P bacteria is to act as an energy reserve during temporary anaerobiosis. For *Acinetobacter* 210A magnesium polyphosphate could also serve as a phosphorus and magnesium source during growth (van Groenestijn et al. 1988, Bark et al. 1992).

Low and high molecule-weight polyphosphates may have a different role in the cell. According to Mino et al. (1984, 1985) low molecule-weight polyphosphates function as an energy source under anaerobic conditions, whereas high molecule-weight polyphosphates may be used as a phosphorus source for growth. The concept of two polyphosphate pools has not been taken into account in the biochemical models of Comeau et al. (1986) and Wentzel et al. (1986).

Cytoplasmic polyphosphates are stored in separate metachromatic subcellular structures often called by the chemically non-specific name of "volutin" granules (Harold 1966). This kind of storage as polymers is a means of maintaining the intracellular charge balance. The volutin granules can be observed in the microscope after staining the cell with basic dyes. In addition to polyphosphates, the volutin granules may contain Mg, Ca, K, ribonucleic acid (RNA) and poly- $\beta$ -hydroxybutyrate. The chemical composition of the granules in *Acinetobacter* and activated sludge have been found to be similar (Heymann et al. 1989).



The size and number of polyphosphate granules can vary with the age of the culture (Streichan et al. 1990), but there can also be strain-specific differences (Bark et al. 1992). More than half of the cell volume can be occupied by polyphosphate granules (Buchan 1983).

In addition to the cytoplasmic polyphosphate pool, there may also be a surface-associated polyphosphate pool in the cell (Halvorson et al. 1987, Hill et al. 1989). In *Acinetobacter lwoffii* Halvorson et al. (1987) found that the surface pool was metabolically more active and disappeared under ATP limitation, for example. Simultaneous release of phosphate was observed. The cytoplasmic pool did not change. The polyphosphates in both pools contained more than 200 phosphate units. The surface pool formed only 1.3 % of the total polyphosphate content of the cell. The results of Hill et al. (1989) also suggest that the orthophosphate released may be involved in the transport of organic matter across the cytoplasmic membrane.

In contrast with the results of Halvorson et al. (1987) and Hill et al. (1989), Streichan and Schön (1991) found polyphosphates only in the cytoplasm of *Acinetobacter* isolates. In *Moraxella* granules were observed outside the cytoplasm as well, namely in the periplasm.

Polyphosphate accumulation seems to be related to the tendency of *Acinetobacter* cells to increase in size and to aggregate into clusters (Buchan 1983). Chains of small cells developed into chains of larger cells, which then disintegrated and formed clusters of very large cells with a high polyphosphate content.

#### 1.4.4 Overplus phenomenon and luxury uptake of phosphate

The specific conditions for accumulation of polyphosphate by bacteria can be divided into three categories (reviewed e.g. by Buchan 1983 and van Groenestijn 1988):

- overplus phenomenon (Liss and Langen 1962)
- luxury uptake of phosphate under unfavourable growth conditions (Harold 1966)
- luxury uptake of phosphate under favourable growth conditions (Fuhs and Chen 1975, Deinema et al. 1980).

The overplus phenomenon takes place when phosphate-starved microorganisms are transferred

into a phosphate-rich medium. The cells immediately take up large amounts of phosphate and store them temporarily as polyphosphates. The overplus phenomenon is likely to take place when the return sludge is mixed with influent wastewater.

The term "luxury" uptake refers to phosphate uptake by growing bacteria in excess to their normal metabolic needs.

Luxury uptake of phosphate under unfavourable growth conditions takes place only in stationary or declining phases of growth. It has been observed in many bacteria. The rate of polyphosphate accumulation is slower than in the overplus phenomenon. This type of luxury uptake is often connected with nutrient imbalance (e.g. nitrogen or sulphur starvation) when nucleic acid synthesis cannot take place.

Luxury uptake under favourable and balanced growth conditions or in the growth phase of bacteria has been observed in some bacteria, e.g. *Acinetobacter* sp. (Fuhs and Chen 1975, Deinema et al. 1980, Hao and Chang 1987) and *Arthrobacter globiformis* isolated from soil (Shoda et al. 1980).

### 1.5 Chemical precipitation during enhanced biological phosphorus removal

It was at first assumed that the spontaneous phosphate removal observed in treatment plants would be mainly due to chemical precipitation reactions (Menar and Jenkins 1970) although there was evidence also on the biological nature of the phenomenon (e.g. Levin and Shapiro 1965).

At present chemical reactions are considered to contribute to enhanced biological phosphorus removal, but they are not believed to be dominating (e.g. Arvin 1979, Ekama et al. 1984, Arvin and Kristensen 1985, Cloete et al. 1985, Miya et al. 1987, Winter 1989, de Haas 1991, de Haas and Greben 1991, Streichan and Schön 1991). Arvin (1983) has divided the biologically mediated phosphate precipitation into two groups: (1) Precipitation in aqueous phase due to high phosphate concentrations after phosphate release from bacteria (2) Precipitation in biofilm due to high pH values inside denitrifying flocs. For calcium phosphate to precipitate the calcium

concentration should be above 50 mg l<sup>-1</sup> Ca and pH above 7.5. Magnesium may also precipitate with phosphorus (Miya et al. 1987).

Streichan and Schön (1991) and Bark et al. (1992) have observed phosphate adsorption and precipitation at the cell surface. However, Buchan (1981) did not find any evidence of calcium phosphate precipitates.

Estimates of the amount of chemical precipitation during biological phosphorus removal vary from zero (Buchan 1981) to 50 - 60 % (Arvin and Kristensen 1985). The highest estimates may be analytical errors as shown by de Haas and Dubery (1989). De Haas (1991) and de Haas and Greben (1991) found chemical precipitation to account for 14 % and 22 % of the total phosphorus removal.

## 1.6 Factors affecting enhanced biological phosphorus removal

Enhanced biological phosphorus removal is affected by many factors, most of which can be derived from the theory of the phenomenon (chapter 1.4). Some physical, chemical and operational factors, which are important from a practical point of view, are presented in the following paragraphs. The subject has been reviewed e.g. by Yeoman et al. (1988) and Toerien et al. (1990).

The physical configuration of an activated sludge plant affects the composition of the microbial population (e.g. Bonting et al. 1992b). The system acts as a selector. Part of the biomass is returned (Fig. 2) to the reactor (e.g. to the aeration basin) after settling out in a clarifier. Flocculated bacteria settle out and are retained in the system (i.e. they are selected for). Bacteria that remain dispersed in the water phase are washed away from the system with the effluent.

It has been suggested that in activated sludge flocculation begins with the formation of species-specific "microflocs", which are then added to the sludge "macroflocs" (Hantula and Bamford 1991). *Acinetobacter* cells have a tendency to form microcolonies (Fuhs and Chen 1975, Buchan 1983, Bark et al. 1992), and the cells, due to their high polyphosphate content, are heavier than others (Suresh et al. 1985). Both of these properties enhance sedimentation of *Acinetobacter* cells

and may be important mechanisms in the enrichment of acinetobacters and other polyphosphate accumulating bacteria in the activated sludge system.

Redox potential is another selective factor. Alternating anaerobic and aerobic zones select for poly-P bacteria, which tolerate, and are thus favoured by these conditions (chapter 1.4.2).

Concentrations of dissolved oxygen in the aerobic zone should be within the range of 2 to 6 mg l<sup>-1</sup>, and definitely not below 1 mg l<sup>-1</sup> anywhere so as to prevent phosphate release in a wrong stage (Yeoman et al. 1988, Toerien et al. 1990). There is a risk of phosphorus release in the settling phase of the sludge as well (Levin and Shapiro 1965).

Phosphate release may also take place in fully aerobic conditions if the aerobic phase is prolonged (Comeau et al. 1987a). The reason for this could be that the carbon storage (PHB, PHV) in the cells becomes depleted before the sludge reaches the end of the aerobic zone, and a new carbon storage begins to build up, resulting in degradation of polyphosphates (chapter 1.4.2).

Biological phosphorus and nitrogen removal are closely linked, both in the positive and negative sense. Nitrate is usually considered to have an adverse effect on the phosphorus removing capacity of activated sludge (Barnard 1974, Mulder and Rensink 1987). The reasons for this are not precisely known. One explanation could be that the presence of nitrate creates an environment where substrates are metabolized through oxidative rather than fermentative pathways. In the treatment plant this would mean that the formation of volatile fatty acids required by the poly-P bacteria in the anaerobic zone is limited (Lötter 1985). Another explanation could be that an anaerobic zone containing nitrate favours denitrifiers, which compete for fermentation products with poly-P bacteria. It should, however, be noted that this reasoning includes the assumption that poly-P bacteria do not denitrify.

The complexity of the nitrate question can be exemplified by the study of Mostert et al. (1988). They studied the anaerobic utilization of volatile fatty acids with sludge treating municipal wastewater. With nitrate present, most of the propionate was used by poly-P bacteria for forming a carbon storage (phosphate was released). Only about 33 to 50 % of acetate and 20 % of

butyrate was used for storing carbon, the rest being consumed by denitrifying bacteria for nitrate reduction. Practically all lactate was utilized for denitrification. Formate did not enhance either carbon storage or denitrification. Acetate and propionate could induce phosphate release under all oxygen-free conditions, but butyrate and lactate could be utilized only under nitrate-free anaerobiosis. Phosphate uptake occurred simultaneously with phosphate release under anaerobic conditions if nitrate was present. Mostert et al. (1988) concluded that this situation might be interpreted erroneously as nitrate inhibiting phosphate release.

To eliminate the potentially harmful effects of nitrate a denitrification zone is often included in the treatment process (chapter 1.7). However, Kuba et al. (1993) have recently reported that denitrification and polyphosphate accumulation may take place simultaneously.

Nitrate and denitrification are important factors to be taken into consideration especially in a nitrogen-removing activated sludge treating municipal wastewater. In forest industry wastewaters the concentration of nitrate is usually low, and there is no or very little nitrification activity in the activated sludge (Jørgensen and Pauli 1992). It is thus unlikely that nitrate would create a problem for enhanced biological phosphorus removal from wastewaters of pulp and paper mills.

Enhanced phosphorus removal is dependent on the presence of readily biodegradable carbon compounds, especially volatile fatty acids. These acids are produced by fermentative bacteria from organic compounds in the influent. Volatile fatty acids are taken up in the anaerobic phase and polymerized at the expense of energy obtained from the breakdown of polyphosphates (chapter 1.4.2). As a result phosphate is released. The release rate thus depends on the rate of fermentation. According to Wentzel et al. (1985) there is a linear relationship between phosphate release and uptake. In practice this chain of interdependent processes means that efficient phosphorus uptake requires high concentrations of fermentable substrates, or volatile fatty acids in the influent to the anaerobic zone.

Generation of fermentation products in primary settling tanks or in special anaerobic reactors, as well as addition of volatile fatty acids to the anaerobic zone have been used as means of

enhancing biological phosphorus removal (Buchan 1982, 1983, Mulder and Rensink 1987, Winter 1989, Abu-ghararah and Randall 1991, Pitman et al. 1992). Abu-ghararah and Randall (1991) studied the effects of various volatile fatty acids. All of them, except formic acid, enhanced significantly both phosphorus release and subsequent uptake. Acetic acid and isovaleric acid enhanced phosphorus removal most efficiently.

Temperatures between 20 and 30 °C appear to be optimal for enhanced biological phosphorus removal, although the process functions also below 10 °C. Within the range of 20 to 30 °C the temperature coefficient  $Q_{10}$  for phosphorus uptake of sludge has been reported to be 2.5 (reviewed by Yeoman et al. 1988). The effect of temperature on polyphosphate accumulation in acinetobacters in activated sludge is variable (van Groenestijn et al. 1989b). The highest growth rates for acinetobacters have been obtained between 25 to 35 °C (Juni 1984, Hao and Chang 1987, van Groenestijn et al. 1989b) or between 20 to 24 °C (Fuhs and Chen 1975). According to Hashimoto and Furukawa (1984) phosphate release from the sludge is dependent on temperature.

Dependence of polyphosphate accumulation on growth rate varies: accumulation may be highest at low growth rates (van Groenestijn et al. 1989b) or increase with increasing growth rates (Hao and Chang 1987).

The pH value for optimum growth of *Acinetobacter* sp. has been found to vary from 6 to 9 (Hao and Chang 1987, van Groenestijn et al. 1989b), depending on the carbon source. Growth has been observed down to pH 4.8 and up to 9.5 (van Groenestijn et al. 1989b). The highest phosphate uptake rate for an *Acinetobacter* isolate studied by Hao and Chang (1987) was at pH 7.0. Van Groenestijn et al. (1989b) found the uptake rate to be independent of pH. Effective phosphorus uptake by sludge has been reported between pH 6 and 8 (Yeoman et al. 1988). Acidic conditions have been found to be necessary to induce phosphorus release in *Acinetobacter lwoffii* (Fuhs and Chen 1975).

The observations on the wide range of temperatures and pH values where phosphate uptake occurs indicate that enhanced biological phosphorus removal can take place under varying conditions, and poly-P bacteria adapted to the prevailing conditions can always be selected for.

Lack of an essential nutrient other than phosphorus or carbon, e.g. nitrogen, may induce accumulation of excess phosphorus, because growth is not possible (luxury uptake under unbalanced growth conditions, chapter 1.4.4). This type of luxury uptake has been observed also in *Acinetobacter* sp. (van Groenestijn et al. 1989b), although for these bacteria luxury uptake of phosphorus under balanced growth conditions is a typical feature.

Certain cations, such as magnesium and potassium, are associated with enhanced phosphorus removal through polyphosphate metabolism as described in chapter 1.4.3. The precise role of these ions in regulation of the activated sludge process is not fully understood (Toerien et al. 1990).

The effects of sulphate and sulphide on enhanced phosphorus removal are poorly known. Experiences with municipal wastewater treatment indicate that sulphide concentrations higher than 25 mg l<sup>-1</sup> S are harmful (Toerien et al. 1990). Sulphide can be toxic and precipitate essential trace minerals. Sulphide is also an energy source for a filamentous bacterium *Thiothrix*, which causes bulking sludge. Presence of sulphate may favour e.g. *Desulfovibrio*, which uses sulphate as an electron acceptor producing sulphide. *Desulfovibrio* competes for certain fatty acids (C<sub>4</sub> and C<sub>5</sub> acids) with poly-P bacteria, but on the other hand it oxidizes them only to acetic acid (Stanier et al. 1975), which is a good substrate for poly-P bacteria.

## 1.7 Treatment plants for enhanced biological phosphorus removal

The term "enhanced biological phosphorus removal" is used here to mean uptake of phosphorus by bacteria in excess to their normal metabolic needs.

Phosphorus removal in the purification of municipal wastewater was investigated already in the 1940s by Jenkins and Locket (1943). Excess phosphate uptake by bacteria in an activated sludge plant was observed for the first time in the 1960s (Levin and Shapiro 1965, Vacker et al. 1967).

There are many different types of treatment plants for enhanced biological nutrient removal.

Some are designed either for nitrogen or phosphorus removal, and some for both. In the following paragraphs some of the best known treatment processes suitable for enhanced biological phosphorus removal are characterized briefly (two modifications of the Bardenpho process called the Five-Stage and Three-Stage Phoredox processes, the UCT process, the Biedenpho process, the Phostrip process and its modification, the Renpho process, the SIBI process). The majority of the laboratory and pilot-scale tests and full-scale applications are from the treatment of municipal wastewaters.

It has been recognized experimentally that anaerobic and aerobic phases are needed and nitrate has an adverse effect on phosphorus removal (Barnard 1974, 1975, 1976, Nicholls 1975). In some cases the anaerobic phase can be generated without any additional constructions by turning off the aerators in part of the aeration basin (e.g. Nicholls and Osborn 1979).

The original **Bardenpho** system with four completely mixed activated sludge basins (Barnard 1974) was designed for nitrogen removal, but in the modified systems efficient phosphorus removal is also possible. In a five-stage modification of the Bardenpho process, also known as the **Five-Stage Phoredox** (Fig. 4), phosphorus removal is optimized by the inclusion of an anaerobic fermentation stage at the beginning of the process (Barnard 1976). The influent and the return sludge are led into this basin. The second and fourth basins do not contain oxygen, but contain nitrate, and the third and fifth basins are aerated. Phosphate uptake, nitrification and carbon oxidation take place in the third, aerobic basin. Part of the mixed liquor from this basin is returned to the first basin, where nitrate is denitrified to nitrogen gas. Another part of the mixed liquor is led directly to the fourth basin, where nitrate is reduced by "endogenous respiration" of the bacteria (i.e. nitrate is denitrified on account of the carbon hydrolyzed from the sludge itself). The mixed liquor is reaerated in the fifth basin to ensure that phosphate is not released in the clarifier.

In the **Three-Stage Phoredox** process the last two basins of the five-stage version have been left out (Ekama et al. 1984). In this process complete denitrification is essential to obtain enhanced phosphorus removal (Ekama et al. 1983).

The so-called **UCT process** (University of

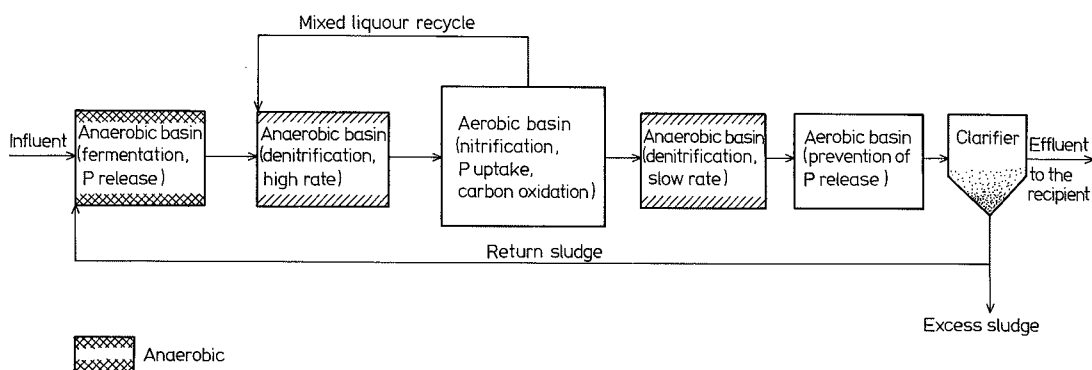


Fig. 4. The Modified Bardenpho process or the Five-Stage Phoredox process.

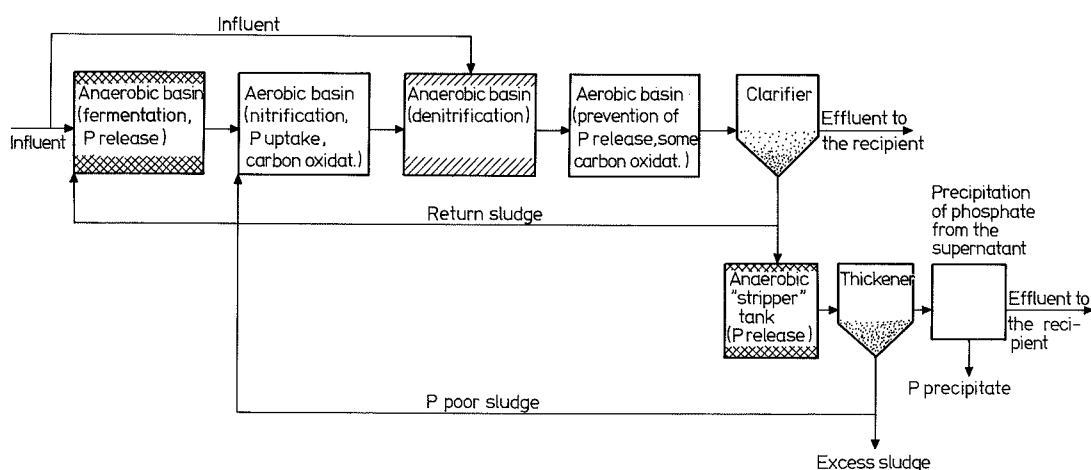


Fig. 5. The Renpho process.

Cape-Town) is similar to the Three-Stage Phoredox process except that the return sludge is discharged to the oxygen free, but nitrate containing reactor instead of the totally anaerobic reactor (Ekama et al. 1983, 1984). In addition there is a flow from the anaerobic nitrate-containing reactor to the anaerobic reactor. In the UCT process complete denitrification is not necessary to achieve enhanced phosphorus removal.

In the **Biodenpho** process there is a separate anaerobic reactor divided in three mixed compartments (Arvin and Kristensen 1985).

The **Phostrip** (Levin and Elster 1985, Levin and Sala 1987) and **Renpho** processes (Rensink et al. 1989) are biological systems combined with

chemical precipitation. Renpho is designed for both phosphorus and nitrogen removal, Phostrip only for phosphorus removal. In the Renpho process (Fig. 5) the return sludge and part of the influent are added to an anaerobic basin, where phosphate is released from the return sludge. In the aerobic basin phosphate is taken up and nitrification occurs. In the following anaerobic, nitrate containing basin denitrification occurs. Part of the influent is added directly to the denitrification basin for carbon source. Denitrifying conditions should inhibit phosphate release in this basin. At the end there is an additional aerobic zone to remove the remaining biodegradable compounds. After settling, the sludge is divided into two parts: one is returned

to the first anaerobic zone, and the other is led to an anaerobic "stripper tank" for phosphate release before being returned to the first aerated zone. Phosphorus is precipitated chemically from this concentrated solution, and removed from the system. Part of the "stripped" phosphorus-poor sludge is returned to the aeration basin for phosphorus uptake.

A modification of the biological phosphorus removal process for treatment of phosphorus-deficient forest industry wastewaters (so-called **SIBI** process) has been developed in Finland (Isoaho et al. 1981, 1985). There is a "stripper tank" in this process as well, but all phosphate released is bound back in biomass as polyphosphates in the contact aeration unit (Fig. 6). Some phosphorus is removed from the system with excess sludge. The polyphosphate containing biomass is recycled to the beginning of the process, where the bacteria use polyphosphates as a phosphorus source to degrade organic matter in the phosphate deficient influent. By recycling part of the phosphorus external phosphorus additions can be minimized or omitted.

One alternative to the traditional enhanced phosphorus removal processes with alternating

aerobic and anaerobic phases would be to maintain a stable enrichment culture of poly-P bacteria separately from the actual activated sludge process. This idea was suggested already by Fuhs and Chen (1975), but it has not been applied in full-scale.

If the sequence of the bacterial genome responsible for polyphosphosphate metabolism can be identified and copied in the future, or if polyphosphate accumulation can be shown to depend on a plasmid (Bayly et al. 1991, Bonting et al. 1992b), genetically modified bacteria could possibly be used for enhanced phosphorus removal under controlled conditions. However, a better understanding of bacterial ecology in activated sludge would also be required. The genes responsible for polyphosphate accumulation and phosphate transport could probably be transferred to the genomes of some dominant sludge bacteria so that the process would not be dependent on the presence of acinetobacters or other polyphosphate bacteria. Kato et al. (1993) have already been able to enhance phosphate accumulation in *Escherichia coli* threefold by modifying the genes involved in the transport and metabolism of phosphate. As

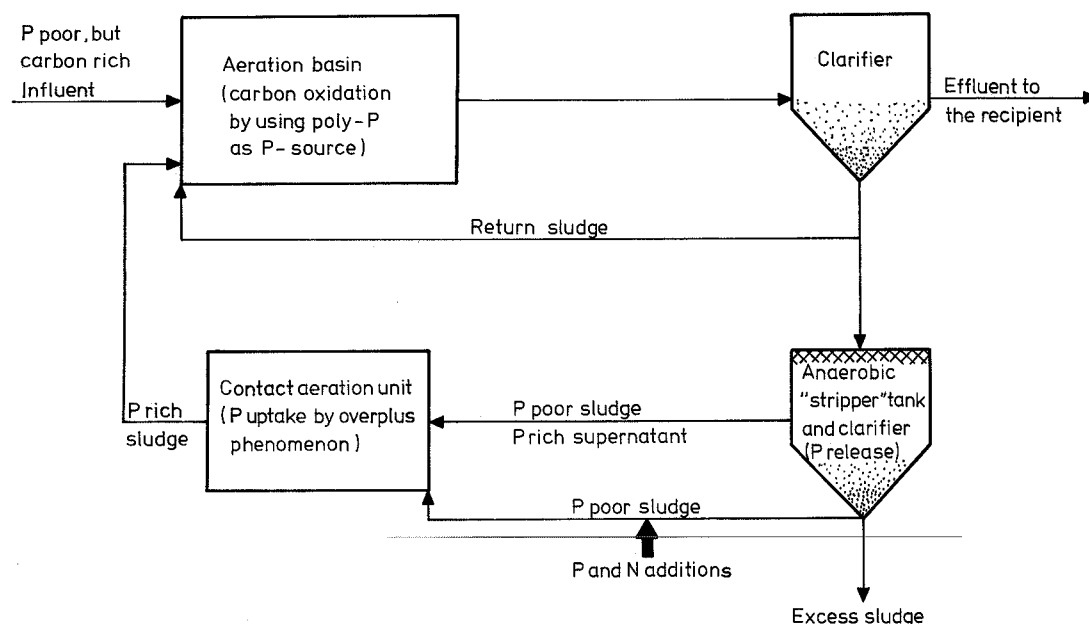


Fig. 6. The SIBI process.

yet there is no information on the maintenance and stability of the modified strains in the sludge.

Enhanced biological phosphorus removal has not been widely accepted, although there are successful applications for the treatment of municipal wastewaters (e.g. a three-stage Bardenpho, van Huyssteen et al. 1990, a modified UCT, Rabinowitz et al. 1990, and other modifications, Hong et al. 1984, Randall et al. 1990, Draaijer et al. 1993, Hansen 1993). Upgrading of existing conventional activated sludge plants for biological phosphorus removal is also feasible at a reasonable expense, which can then be recovered through savings in operational costs (Randall et al. 1990).

Because of their sophisticated biological processes, nutrient removal plants require better monitoring and control than conventional treatment plants (Pitman 1991). This may be one of the reasons why these processes have not been widely implemented by municipalities and industry. If the reasons for malfunctions remain obscure the prospects of controlling the process are limited.

In Finland there is one full-scale plant treating wastewater from a sulphate pulp mill with the SIBI process (Luonsi et al. 1986, Mehner and Silvo 1988), but no full-scale municipal treatment plant that would remove phosphorus only biologically, without chemicals. A pilot-scale experiment on enhanced biological phosphorus removal in municipal wastewater was started in 1993 by the National Board of Waters and the Environment.

## 1.8 Objectives of the study

Neither the amenability of forest industry wastewaters to enhanced biological phosphorus removal nor the phosphorus-removing potential of the activated sludge treating them has been studied earlier from a microbiological point of view.

There are no previous studies on the number of acinetobacters or other polyphosphate accumulating bacteria in sludge treating wastewaters from pulp and paper mills. As reviewed in chapter 1.4 estimates on the occurrence and significance of acinetobacters in municipal sludge with enhanced biological phosphorus removal

vary in different studies. In Finland most of the forest industry wastewaters are treated by an aerobic activated sludge process which, on the basis of the biochemical models presented in the literature, is not likely to enrich acinetobacters. If enhanced biological phosphate removal is to be considered as an option for the conventional aerobic process in the treatment of forest industry wastewaters as well, it is important to know whether acinetobacters even belong to the normal microbial flora of the sludge in the present activated sludge processes or to the flora of the influent wastewaters.

Among the factors known to be important for growth and polyphosphate accumulation of acinetobacters are concentrations of volatile fatty acids, as well as concentrations of potassium and magnesium in the growth medium. The nitrogen to phosphorus ratio in the growth medium may have an effect as well. The literature presents very little quantitative data on the effects of these factors on the growth of acinetobacters. Information on the concentrations of volatile fatty acids, potassium and magnesium in forest industry wastewaters is scarce as well.

In the biochemical models describing excess phosphate uptake by acinetobacters, aerobic and anaerobic phases are considered to be necessary. Phosphate uptake occurs under aerobic conditions, and phosphate release is thought to require anaerobic conditions. Information available on the factors affecting phosphate release is not always consistent. Furthermore, there are not many studies presenting kinetic models for the phosphate uptake of acinetobacters. Uptake kinetics and the number of acinetobacters should, however, be known when estimating the significance of acinetobacters in phosphorus removal.

The question of the denitrification potential of acinetobacters is of interest when simultaneous phosphorus and nitrogen removal is aimed at. There are contradictory results reported in the literature on the denitrification ability of acinetobacters.

The purpose of this investigation was

- \* to obtain information on bacterial populations in forest industry wastewaters and activated sludge treating these wastewaters paying special attention to acinetobacters, which are considered as model organisms for polyphosphate accumulating bacteria,

- \* to study how the growth of acinetobacters could be optimized with respect to the nitrogen-phosphorus ratio and magnesium and potassium levels in the medium,
- \* to study the phosphate uptake and release kinetics of acinetobacters (and for comparison, three other bacteria from sludge),
- \* to estimate the significance of acinetobacters in the phosphorus removal of the present aerobic activated sludge processes in forest industry
- \* to study the denitrification potential of acinetobacters.

## 2 MATERIALS AND METHODS

### 2.1 Treatment plants studied

Samples of wastewater and activated sludge were obtained from the activated sludge treatment plants of two pulp mills and three paper mills. The treatment plants were chosen so as to represent different types of pulping or papermaking wastewater. Moreover, availability of earlier studies of the treatment plants and coordination with other ongoing studies in this field (e.g. Jørgensen and Pauli 1992, Lammi and Pakarinen 1993, Puustinen 1993) affected the choice of treatment plants.

A short description of the production and wastewater treatment processes at the mills studied, based on the information given by the mills (collected by Puustinen and Jouttijärvi, unpublished data), is given below. Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) are standard parameters used in wastewater engineering (U.S. Environmental Protection Agency, 1975, American Public Health Association, 1989). The parameter MLSS gives an estimate of the dry matter content of the sludge. It includes all material that remains after evaporating the sample for 24 h at 105 °C. The parameter MLVSS means the weight lost in the ignition of the dried sample to constant weight at 550 °C, i.e. the ignition loss (the material that is "volatilized"). The abbreviations

SC stands for uncoated paper, and LWC for light-weight coated paper.

**Pulp mill 1** produced chlorine-bleached sulphate pulp and served as an example of a kraft pulp mill. The treatment plant was new. It received wastewaters from pulping and debarking processes. Acidic wastewater fractions were neutralized with lime mud. The sludge load was 0.15 kg BOD kg<sup>-1</sup> MLSS d<sup>-1</sup>, sludge age 15 d and retention time 24 h. Nitrogen was added as urea 190 – 235 kg d<sup>-1</sup> N (1.5 – 2.0 g m<sup>-3</sup> N) to the wastewater before aeration. Phosphorus was not added, since the lime mud contains phosphorus.

**Pulp mill 2** produced mainly bleached sulphate pulp and some paper as well. Wastewaters from pulp and paper making processes were discharged into the treatment plant, where the wastewater was neutralized before pre-aeration. The sludge load was 0.15 – 0.20 kg BOD kg<sup>-1</sup> MLSS d<sup>-1</sup>, sludge age 14 d and retention time 12 h. No nutrients were usually added, but there were test periods when nitrogen was added (280 kg d<sup>-1</sup> N) before the wastewater reached the equalization basin. The wastewater was retained in the equalization basin for six hours before it was led to the aeration basin. The sample of the influent wastewater was taken after the equalization basin.

**Paper mill 1** produced wood-containing fine papers (SC and LWC). The treatment plant received wastewaters from mechanical pulp and paper production and debarking. The sludge load was 0.20 – 0.25 kg BOD kg<sup>-1</sup> MLVSS d<sup>-1</sup>, sludge age 8 – 11 d and retention time 16 h. Both nitrogen, as urea, and phosphorus, as phosphoric acid, were added. The additions varied at short intervals from 150 to 590 kg d<sup>-1</sup> N (averaging 400 kg d<sup>-1</sup> N or 36 g m<sup>-3</sup> N) and from 0 to 63 kg d<sup>-1</sup> P (averaging 23 kg d<sup>-1</sup> P or 2 g m<sup>-3</sup> P). Nitrogen was added to the neutralized influent and phosphorus to the return sludge immediately before it was fed to the aeration basin.

**Paper mill 2** produced wood-containing paper (SC and LWC) and small amounts of paper containing recycled fibres. The treatment plant received wastewaters from mechanical pulp and paper production and debarking, as well as small quantities of coating components. The sludge load was 0.15 kg BOD kg<sup>-1</sup> MLVSS d<sup>-1</sup>, sludge age 10 d and retention time 19 h. The wastewater was neutralized and spiked with urea (3.3 g m<sup>-3</sup> N) before it was led to the equalization basin.



Phosphorus was not added.

Paper mill 3 produced newsprint paper. The treatment plant received wastewaters from mechanical pulp and paper production and debarking. The sludge load was  $0.3 - 0.6 \text{ kg BOD kg}^{-1} \text{ MLSS d}^{-1}$ , sludge age  $3 - 5 \text{ d}$  and retention time  $12 \text{ h}$ . Nutrients were added to the return sludge immediately before aeration. Nitrogen was added as urea  $248 \text{ kg d}^{-1} \text{ N}$  ( $12.4 \text{ g m}^{-3} \text{ N}$ ), and phosphorus as phosphoric acid  $61.5 \text{ kg d}^{-1} \text{ P}$  ( $3.1 \text{ g m}^{-3} \text{ P}$ ).

Average nitrogen and phosphorus concentrations in the influents before and after nutrient additions, if made, and in the purified effluent of the studied treatment plants are given in Table 3 (Puustinen and Jouttijärvi, unpublished data). Phosphate concentrations in the influents to the treatment plants ranged from  $1$  to  $4 \text{ mg l}^{-1} \text{ P}$ .

## 2.2 Sampling

Samples were taken between January 1991 and April 1992. Samples for the physical and chemical analyses and microbiological studies were taken from the influent wastewater, at the beginning and end of the aeration basin, and from the return sludge. In some cases the sample to represent the influent was taken from the equalization basin. The sample of the influent was taken after neutralization and nutrient additions whenever technically possible.

The samples were taken into clean plastic bottles. Space for air was left in them, except for samples to be analyzed for volatile fatty acids and organic carbon. These bottles were filled completely to minimize evaporation of the volatile compounds.

Table 3. Average concentrations of nitrogen (N) and phosphorus (P) in the influent to the treatment plant before and after (INF) nutrient addition(s), and in the purified wastewater (EFF). The data is from the years 1991 and 1992 (Puustinen and Jouttijärvi, unpublished data).

Treatment plant	Concentration ( $\text{mg l}^{-1}$ )					
	Total N	Soluble N	$\text{NH}_4^+-\text{N}$	Total P	Soluble P	$\text{PO}_4^{3-}-\text{P}$
<b>Pulp mill 1</b>						
Before add.	2.9	..a)	0.2	1.4	1.0	0.8
INF (+N)	..	5.5	1.0	..	..	0.8
EFF	2.6	1.5	0.2	0.8	0.5	0.5
<b>Pulp mill 2</b>						
Before add.	4.1	..	..	1.5	..	1.0
INF	..	..	..	..	..	1.0
<b>Paper mill 1</b>						
Before add.	10.6	9.8	0.8	3.2	..	2.0
INF (+N+P)	46.0	36.8	1.5	5.0	..	4.0
EFF	5.5	4.6	0.1	0.8	0.5	0.4
<b>Paper mill 2</b>						
Before add.	4.0	3.5	..	1.5	0.7	0.6
INF (+N)	14.6	..	..	1.4	<0.1	0.6
EFF	4.6	2.2	0.1	0.5	<0.1	0.0
<b>Paper mill 3</b>						
Before add.	6.4	5.7	..	2.6	2.3	1.5
INF (+N+P)	18.0	..	..	4.4	..	..
EFF	4.9	..	..	1.3	0.6	0.6

a) No data

Samples for chemical analyses were filtered immediately after sampling through an Acrodisc PF syringe filter with a 0.8  $\mu\text{m}$  prefilter and a 0.2  $\mu\text{m}$  final filter (product number 4187, Gelman Sciences). The filtering removes microorganisms and stops biological activity.

The bottles with the air space were transported horizontally to ensure proper aeration of the samples.

There was a delay of one to five hours between sampling and analyses. The temperature of the samples was approximately 30 °C, but it was lowered during transport. This may have resulted in some unknown changes in the bacterial populations.

### 2.3 Physical and chemical analyses of wastewater and activated sludge

The concentration of dry matter and the ignition loss (representing organic matter) in the influent wastewater and activated sludge were determined according to standard SFS 3008.

Filtrates of influent wastewater and sludge were analyzed by atomic absorption spectroscopy (atomization in flame) for concentrations of calcium ( $\text{Ca}^{2+}$ ) and magnesium ( $\text{Mg}^{2+}$ ) according to standard SFS 3018 and for potassium ( $\text{K}^+$ ) according to standard SFS 3017.

Concentrations of volatile fatty acids (formic, acetic, propionic and butyric acids) were analyzed in filtered samples by using an ionchromatograph equipped with a Millipore Waters ion exclusion column (7.8 · 300 mm) or by using gas chromatography after methylation (one set of samples from pulp mill 1 was analyzed without methylation after extraction with diethyl ether).

Concentrations of dissolved organic carbon (DOC) were analyzed in filtered samples with Easy Quant Universal Carbon Analyzer EQ 90 (Easy Quant) after acidification (10  $\mu\text{l}$  7 N  $\text{H}_3\text{PO}_4$ /1.0 ml of sample).

### 2.4 Cultivation, enumeration, isolation and identification of bacteria in wastewater and activated sludge

Aerobic, heterotrophic bacteria in influent wastewaters and activated sludge were studied in

the treatment plants of pulp mill 2 and paper mills 1, 2 and 3.

Bacteria in the treatment plant of pulp mill 2 were studied twice within a 2-month period, paper mill 1 four times within a 12-month period, paper mill 2 three times within a 5-month period and paper mill 3 twice within a 6-month period.

The samples (diluted 1:10 with sterilized 0.9 % saline) were homogenized by mixing them with Tween 80 (100  $\mu\text{l}$  2 % Tween 80/100 ml of sample) and tripolyphosphate (1.0 ml/100 of sample; 1.04 g  $\text{Na}_3\text{P}_3\text{O}_{10}$ /10 ml distilled water). The mixture was blended for 2 min, cooled in ice for 1 min (repeated) and finally blended for 1 min. According to preliminary experiments this procedure gives higher counts of colony forming units (CFU  $\text{ml}^{-1}$ ) than the other homogenization methods tested (Jørgensen and Puustinen, unpublished results).

Total colony counts, as colony forming units of heterotrophic, aerobic bacteria, were determined on tryptone-glucose-yeast (diluted 1:5) agar (Difco) plates (TGY 1:5 plates) by spreading suitable dilutions (in 0.9 % saline) of the homogenized wastewater or activated sludge on the plates. Diluted TGY medium is commonly used in the cultivation of bacteria in wastewater and sludge (American Public Health Association, 1989). There is, however, no single medium that would sustain the growth of all aerobic, heterotrophic bacteria at the same time. By comparing different enumeration methods it has been estimated that less than 14 % of the bacterial population of the sludge can be cultivated (e.g. Unz and Dondero 1970, Cloete and Steyn 1987). In spite of its shortcomings plating is the only practicable enumeration method for routine use if bacteria are also to be isolated for identification.

Acinetobacters were cultivated on an acetate mineral medium. The acetate mineral medium was composed on the basis of a commonly used recipe by Deinema et al. (1980), modified by Jørgensen, Puustinen and Kaitala (unpublished results). The modification was based on the results of factorial experiments designed to find a combination of carbon, nitrogen, phosphorus, magnesium and potassium (nutrients known to be significant for polyphosphate bacteria) that would give maximum growth for two unidentified bacteria isolated from activated sludge

(isolates 900026 and 900030).

The acetate mineral medium had the following composition (concentrations of the main nutrients are given as elements): 650 mg l<sup>-1</sup> C (as CH<sub>3</sub>COONa · 3H<sub>2</sub>O), 5 mg l<sup>-1</sup> P (as Na<sub>2</sub>HPO<sub>4</sub> · 2H<sub>2</sub>O), 15 mg l<sup>-1</sup> N (as NH<sub>4</sub>Cl), 13 mg l<sup>-1</sup> Mg (as MgSO<sub>4</sub> · 7H<sub>2</sub>O), 12 mg l<sup>-1</sup> K (as K<sub>2</sub>SO<sub>4</sub>), 4.7 mg l<sup>-1</sup> Ca (as CaCl<sub>2</sub> · 2H<sub>2</sub>O), 12 g l<sup>-1</sup> HEPES-buffer (pK<sub>a</sub> = 7.55) and agar 15 mg l<sup>-1</sup> (Bacto, Difco). In addition, 2.0 ml of a trace mineral solution with the following composition (adapted from van Groenestijn 1988) was added per one litre of the medium: 50 g l<sup>-1</sup> EDTA, 5 g l<sup>-1</sup> FeSO<sub>4</sub> · 7H<sub>2</sub>O, 1.6 g l<sup>-1</sup> CuSO<sub>4</sub> · 5H<sub>2</sub>O, 5 g l<sup>-1</sup> MnCl<sub>2</sub> · 4H<sub>2</sub>O, 1.1 g l<sup>-1</sup> (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub> · 4H<sub>2</sub>O, 50 mg l<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>, 10 mg l<sup>-1</sup> KI, 50 mg l<sup>-1</sup> CoCl<sub>2</sub> · 6H<sub>2</sub>O. All reagents were pro analysis quality (Merck). The pH value was adjusted to 7.0 with 1 M NaOH.

The media were sterilized by autoclaving (1 bar, 121 °C for 15 min.) All glassware was acid-washed (soaked 24 h in 2 M HCl, rinsed 3 – 4 times with tap water and once with distilled water, standard SFS 3025) and sterilized.

The plates were incubated at 30 °C. This temperature is close to the average temperature in the treatment plants (Jørgensen and Pauli 1992) and commonly used for cultivating sludge bacteria, including acinetobacters (Baumann 1968, Suresh et al. 1985, Hiraishi 1988, Beacham et al. 1990, Bayly et al. 1991).

The colonies were counted after 2 – 3 d (fast growing bacteria) and after 5 – 7 d (slowly growing bacteria) of incubation. Incubation time of 2 d is often used for cultivation of acinetobacters in the sludge (e.g. Baumann 1968, Suresh et al. 1985).

The concentration of bacteria (i.e. CFU ml<sup>-1</sup>) was calculated with 95 % confidence limits after testing the consistency of the colony counts in different dilutions with the  $\chi^2$  test as described e.g. by Niemelä (1977).

In order to get an estimate of the number of acinetobacters growing on the acetate mineral medium, colonies on the most diluted plates after a 2 d incubation period were chosen at random for purification and identification with API 20 NE (Non-Enteric) identification kit (bioMérieux sa). The fast-growing bacteria were likely to be acinetobacters, growing well on the acetate mineral medium. Colonies on the TGY medium were not identified.

For purification the colonies were streaked twice on the acetate mineral medium and at least once on the TGY (1:5) medium. The purity of the colonies was checked by microscopical examination of the Gram stained smears before identification.

The API identification system has been designed for non-enteric, Gram-negative rods. It is based on testing 21 physiological traits, which include nine biochemical traits and the ability to assimilate aerobically 12 different compounds as sole carbon sources. The biochemical reactions of the isolate under study are compared with the biochemical profiles in the data base. The percentage of identification (%id) gives an estimate of how closely the obtained profile corresponds to the taxon relative to all the other taxa in the data base. The T index gives an estimate of how closely the isolate profile corresponds to the most typical set of reactions for each taxon. Its value varies between 0 and 1 and is inversely proportional to the number of atypical tests. The quality of identification is determined by the percentage of identification and the T value (Instruction Manual, Apilab Plus, bioMérieux sa):

Excellent identification %id ≥ 99.9 and T ≥ 0.75  
 Very good identification %id ≥ 99.0 and T ≥ 0.5  
 Good identification %id ≥ 90.0 and T ≥ 0.25  
 Acceptable identification %id ≥ 80.0 and T ≥ 0

In this study identification results falling into the classes above were considered acceptable. Isolates identified with the comment "low discrimination", "doubtful profile" or "unacceptable profile" were considered as unidentified.

Although acetate mineral medium selects for acinetobacters some other bacteria grow on it as well. The number of acinetobacters was estimated by correcting the number of colony forming units on the acetate mineral medium by the percentage of colonies identified as acinetobacters.

From the influent and activated sludge of pulp mills 60 bacterial isolates were identified and from paper mills 218 isolates. The isolates were preserved in glycerol. They are now stored at -70 °C in the microbiological laboratory of the National Board of Waters and the Environment.

## 2.5 Enrichment of acinetobacters

Acinetobacters were enriched from samples taken from treatment plants of pulp mill 1 (72 bacterial isolates from two sampling times) and paper mill 1 (22 bacterial isolates from two sampling times) by using a liquid acetate mineral medium of the same composition as described in chapter 2.4.

The enrichment procedure was essentially the same as the method introduced by Baumann (1968). For enrichment 5 or 10 ml of activated sludge was suspended in 95 or 90 ml of the acetate mineral medium (i.e. 5 or 10 % inocula). This culture was incubated aerobically in an Erlenmeyer flask on a rotary shaker (100 rpm) for 2 – 3 d. An aliquot of 5 or 10 ml of this culture was transferred further to 95 or 90 ml of a fresh acetate mineral medium and incubated as described above. These transfers were made two or three times, resulting in an enrichment time of 4 to 7 days.

A dilution series of the enrichment culture was cultivated on the acetate mineral medium (recipe in chapter 2.4), from which colonies were picked randomly, purified and identified as described in chapter 2.4.

Incubation temperature of both the enrichment cultures and the plates was 30 °C except for the first enrichment, where the temperature was adjusted to 32 °C in accordance with the *in situ* temperature in the aeration basin (*A. baumannii*, isolate 910045 was isolated then). The plates were incubated for 3 – 5 d.

## 2.6 Staining of polyphosphate granules

The presence of polyphosphate granules was examined by using the Neisser stain (Gurr 1965). The granules are dark violet spots often situated

at the ends of the rod-shaped cells. Other polymeric compounds such as poly- $\beta$ -hydroxybutyrate (a polymerization product of acetic acid) react with this stain to produce opaque, refractive bodies (Martinez 1963, Bonting et al. 1992a).

## 2.7 Optimization of growth conditions for three *Acinetobacter* isolates

Three *Acinetobacter* pure cultures (Table 4) were used as model organisms for polyphosphate accumulating bacteria (poly-P bacteria). *A. baumannii* and *A. junii* were isolated from enrichment cultures of activated sludge samples taken at the beginning of the aeration basin of pulp mill 1. At the time of sampling (24.1.1991 and 14.3.1991) the temperatures in the aeration basin were 32.5 °C and 31 °C, respectively. The isolate of *A. lwoffii* was obtained from K.S. Jørgensen and J. Puustinen. It was isolated from the return sludge of paper mill 1. The isolation procedure was basically the same as described in chapter 2.4.

The percentages of identification and T values for the *Acinetobacter* isolates obtained by the API identification system were good or excellent (Table 4). All three isolates were able to accumulate phosphate (c.f. chapter 3.5).

The isolates were maintained on the TGY (1:5) medium at 4 °C, and recultivated at least once a month. A new inoculum was recultured regularly from the stock culture kept at –70 °C.

The effects of various concentrations of C, N, P, Mg and K (Table 5) on the growth of acinetobacters were studied by using four sets of factorial design experiments (Box and Wilson 1951, Bayne and Rubin 1986). There were two (Mg, K), three (C, N, P and N, P, Mg) or four (P, N, Mg, K) factors (or variables) in one experiment. Low (coded –1) and high (coded +1)

Table 4. Identifications (API 20 NE identification kit) and origins of the three *Acinetobacter* isolates used as model organisms for polyphosphate accumulating bacteria.

Isolate	Identification %	T value	Origin
<i>A. lwoffii</i> , 9003	98.2 (good)	0.69	return sludge, paper mill 1
<i>A. baumannii</i> , 910045	99.9 (excellent)	0.99	enrichment culture of activated sludge, pulp mill 1
<i>A. junii</i> , 910093	92.0 (good)	0.99	enrichment culture of activated sludge, pulp mill 1

Table 5. Compounds and concentration ranges in the medium used in the growth optimization tests of acinetobacters.

Compound	Element	Concentration	
		(mg l <sup>-1</sup> )	(mmol l <sup>-1</sup> )
CH <sub>3</sub> COONa · 3H <sub>2</sub> O	C	166.0 – 500.0	13.8 – 41.6
NH <sub>4</sub> Cl	N	15.0 – 187.0	1.1 – 13.4
Na <sub>2</sub> HPO <sub>4</sub> · 2H <sub>2</sub> O or Na <sub>2</sub> HPO <sub>4</sub> + NaH <sub>2</sub> PO <sub>4</sub> · H <sub>2</sub> O	P	1.0 – 30.0	0.032 – 0.97
MgSO <sub>4</sub> · 7H <sub>2</sub> O	Mg	1.0 – 15.0	0.082 – 0.62
K <sub>2</sub> SO <sub>4</sub>	K	2.0 – 22.0	0.051 – 0.56

concentrations and the average of these, called the centre points (coded 0; having two or more replicates) were chosen for each factor (Fig. 7). Each factor had all combinations of the low and high levels. In experiments 3 and 4 so-called star points (coded -2 and +2) were also included. The steps between the concentration levels coded with -2, -1, 0, +1, +2 are equal. For instance in the third experiment the low level for factor P was 4 mg l<sup>-1</sup> P (-1), the high level was 10 mg l<sup>-1</sup> P (+1), the average was 7 mg l<sup>-1</sup> P (0; 13 replicates), and the star points were 1 mg l<sup>-1</sup> P (-2) and 13 mg l<sup>-1</sup> P (+2). The step was then 3 mg l<sup>-1</sup> P.

Coding was used to obtain non-correlated treatments for regression calculations. This makes it possible to reveal the combined effects of the treatments. In these experiments the principle of evolutionary operations (Box 1957) was followed,

i.e. the design of an experiment was based on the information obtained in the one preceding it. In this way combinations of concentrations giving maximum growth were gradually approached.

The four factorial experiments were the following:

Experiment 1. Concentrations of C, N and P were varied (factorial design: 2<sup>3</sup> and the centre points)

Experiment 2. Concentrations of N, P and Mg were varied (factorial design: 2<sup>3</sup> and the centre points)

Experiment 3. Concentrations of P, N, Mg and K were varied (factorial design: 2<sup>4</sup>, the centre points and the star points)

Experiment 4. Concentrations of Mg and K were varied (factorial design: 2<sup>2</sup>, the centre points and the star points)

The design matrix for experiment 1 can be presented ( $x_1$ ,  $x_2$  and  $x_3$  are the coded values, and C, N, P the real values for the factors studied) as follows:

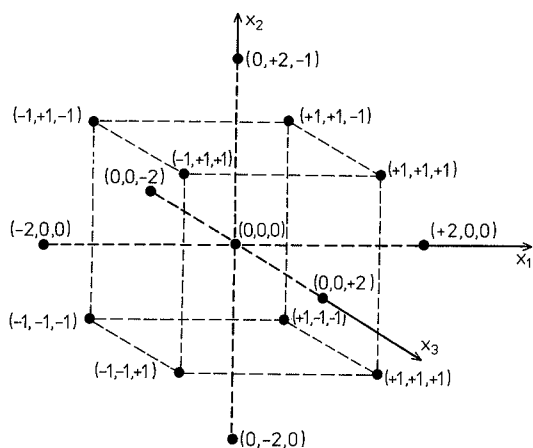


Fig. 7. The experimental region in a three-factor design. The levels are coded with 0 (centre point), +1, -1 (high and low levels) and +2, -2 (the star points). The coordinates are presented in the order  $x_1$ -,  $x_2$ - and  $x_3$ -axis.

Experimental unit	$x_1$	$x_2$	$x_3$	C (mg l <sup>-1</sup> )	N (mg l <sup>-1</sup> )	P (mg l <sup>-1</sup> )
1	-1	-1	-1	166	46	10
2	1	-1	-1	500	46	10
3	-1	1	-1	166	140	10
4	1	1	-1	500	140	10
5	-1	-1	1	166	46	30
6	1	-1	1	500	46	30
7	-1	1	1	166	140	30
8	1	1	1	500	140	30
9	0	0	0	333	93	20
10	0	0	0	333	93	20

The design matrix for experiment 2 can be presented ( $x_1$ ,  $x_2$  and  $x_3$  are the coded values, and N, P, Mg the real values for the factors studied) as follows:

Experimental unit	$x_1$	$x_2$	$x_3$	N (mg l <sup>-1</sup> )	P (mg l <sup>-1</sup> )	Mg (mg l <sup>-1</sup> )
1	-1	-1	-1	93	15	5
2	1	-1	-1	187	15	5
3	-1	1	-1	93	30	5
4	1	1	-1	187	30	5
5	-1	-1	1	93	15	15
6	1	-1	1	187	15	15
7	-1	1	1	93	30	15
8	1	1	1	187	30	15
9	0	0	0	140	22.5	10
10	0	0	0	140	22.5	10

The design matrix for experiment 3 can be presented ( $x_1$ ,  $x_2$ ,  $x_3$  and  $x_4$  are the coded values, and P, N, Mg, K the real values for the factors studied) as follows:

Experimental unit	$x_1$	$x_2$	$x_3$	$x_4$	P (mg l <sup>-1</sup> )	N (mg l <sup>-1</sup> )	Mg (mg l <sup>-1</sup> )	K (mg l <sup>-1</sup> )
1	-1	-1	-1	-1	4	40	4	4
2	1	-1	-1	-1	10	40	4	4
3	-1	1	-1	-1	4	90	4	4
4	1	1	-1	-1	10	90	4	4
5	-1	-1	1	-1	4	40	8	4
6	1	-1	1	-1	10	40	8	4
7	-1	1	1	-1	4	90	8	4
8	1	1	1	-1	10	90	8	4
9	-1	-1	-1	1	4	40	4	8
10	1	-1	-1	1	10	40	4	8
11	-1	1	-1	1	4	90	4	8
12	1	1	-1	1	10	90	4	8
13	-1	-1	1	1	4	40	8	8
14	1	-1	1	1	10	40	8	8
15	-1	1	1	1	4	90	8	8
16	1	1	1	1	10	90	8	8
17	-2	0	0	0	1	65	6	6
18	2	0	0	0	13	65	6	6
19	0	-2	0	0	7	15	6	6
20	0	2	0	0	7	115	6	6
21	0	0	-2	0	7	65	2	6
22	0	0	2	0	7	65	10	6
23	0	0	0	-2	7	65	6	2
24	0	0	0	2	7	65	6	10
25	0	0	0	0	7	65	6	6
26	0	0	0	0	7	65	6	6
27	0	0	0	0	7	65	6	6
28	0	0	0	0	7	65	6	6
29	0	0	0	0	7	65	6	6
30	0	0	0	0	7	65	6	6
31	0	0	0	0	7	65	6	6

The design matrix for experiment 4 can be presented ( $x_1$ ,  $x_2$  are the coded values, and Mg, K the real values for the factors studied) as follows:

Experimental unit	$x_1$	$x_2$	Mg (mg l <sup>-1</sup> )	K (mg l <sup>-1</sup> )
1	-1	-1	5	7
2	-1	1	5	17
3	1	-1	11	7
4	1	1	11	17
5	-2	0	2	12
6	2	0	14	12
7	0	-2	8	2
8	0	2	8	22
9	0	0	8	12
10	0	0	8	12
11	0	0	8	12
12	0	0	8	12

The carbon concentration in optimization experiments 2 – 4 was fixed to a level that does not limit growth even at a phosphorus concentration of 30 mg l<sup>-1</sup> P and nitrogen concentration of 140 mg l<sup>-1</sup> N (the high levels of the nutrient concentrations). This level was determined in the first experiment with *Acinetobacter baumannii*, isolate 910045 as a model organism. A non-limiting carbon concentration was reached when the addition of carbon no longer resulted in an increase of biomass (c.f. Brown and Cooper 1991). The optimal carbon concentration was obtained by curve-fitting and derivation using the optical density (540 nm) of a stationary phase culture (after 23 h) as a measure of bacterial biomass.

In addition to the compounds tested, 3 mg l<sup>-1</sup> Ca and 2.0 ml of the trace mineral solution described in chapter 2.4 were added per litre of the growth medium. The medium was buffered to pH 7.0 with HEPES-buffer (6 – 9 g l<sup>-1</sup>).

Experiments 1 and 2 were performed both manually in Erlenmeyer flasks (shaking 100 rpm) and automatically with a Bioscreen C analyzer (LabSystems). The parallel tests were run to check if the cell suspensions were properly aerated by the Bioscreen C analyzer. Since the two methods gave comparable results, tests 3 and 4 were run only with the Bioscreen C analyzer.

For the optimization experiments the bacterial inocula were prepared in the following manner: a few colonies were picked from the TGY medium and transferred to 20 ml of the acetate mineral medium (recipe in chapter 2.4) and incubated on a rotary shaker (100 rpm) at 30 °C for about 24 h. A volume of 10 ml of this culture was transferred to 100 ml of fresh acetate mineral medium and incubated overnight. This culture was then used to inoculate the experimental bottles or Bioscreen wells. The volume of the inoculum was 2 % in the manual tests and 6 % in the Bioscreen tests of the volume of the growth medium. The inoculation percentage for the Bioscreen was chosen based on experiments with different volumes of inocula (1.5 – 8.8 %).

Bacterial growth was measured by monitoring changes in the optical densities (540 nm in manual and 600 nm in Bioscreen experiments) of the bacterial suspensions. The cultures were incubated at 30 °C for 23 h or until they had reached a stationary phase of growth.

All glassware used for the experiments was

acid-washed (standard SFS 3025) and sterilized.

The results were analyzed with a Matlab software package for numeric computations, version 3.5 (Matlab for Windows 1991). Significant effects were calculated by using the probability values and normal probability plots (Box and Draper 1987).

## 2.8 Phosphate uptake kinetics of four *Acinetobacter* isolates and three other isolates

Phosphate uptake kinetics of polyphosphate accumulating bacteria was studied by using four *Acinetobacter* isolates as model organisms (Table 6). Three of the isolates were the same as those used in the growth optimization experiments.

For comparison, phosphate uptake kinetics was also studied in three other bacteria isolated from the treatment plants on the acetate mineral medium (Table 6). It was assumed that bacteria capable of growing on the acetate mineral medium would have some characteristics in common, e.g. the ability to accumulate excess phosphate. This was indicated by the ability of these bacteria to form granules visible in the microscope after dyeing with the Neisser stain, which is supposed to be specific for polyphosphate.

All glassware used for the experiments was acid-washed (standard SFS 3025) and sterilized. Incubations were made either in ordinary Erlenmeyer flasks or in flasks with a side arm (Bellco) which could be used for readings of optical density without subsampling.

At pH values exceeding 7.5 orthophosphate may

precipitate with calcium, magnesium and ammonium (Snoeyink and Jenkins 1980, Arvin 1983, Miya et al. 1987). In actively growing *Acinetobacter* cultures pH may rise above 7.5 in spite of buffering. To check whether chemical precipitation or physical adsorption of orthophosphate to the glassware took place within an incubation time of 24 h and at pH values up to 8.7 the following test was made. The sterile liquid acetate mineral medium (so-called phosphate uptake medium) with a phosphate concentration of 8 mg l<sup>-1</sup> P (for a complete recipe see "phosphate uptake by a growing culture") was incubated without bacteria, but otherwise following the same procedures as in the phosphate uptake experiments. There were two sets of experiments: (1) the ordinary phosphate uptake medium with the trace mineral solution, i.e. containing the chelating agent EDTA and (2) the same medium without the EDTA-containing trace mineral solution. The pH values of both media were adjusted to 7.5 and 8.7 with 1 M NaOH. Orthophosphate concentrations were measured before the pH adjustments and incubation and again after incubating the media for 4 and 24 h. The samples were filtered through a 0.2 µm syringe filter (Acrodisc PF) before analyses to remove any possible precipitation.

Phosphate uptake kinetics was studied in two different ways. The first approach was based on a short-term incubation of bacteria in a batch culture. In these experiments growth was negligible, and all or most of the phosphate uptake was in excess to the normal metabolic phosphorus requirements of the cell. In the second approach phosphate uptake was followed during bacterial growth in a batch culture, i.e.

Table 6. Identifications (API 20 NE identification kit) and origins of the isolates used in the phosphate uptake experiments.

Isolate	Identification %	T value	Origin
<i>A. lwoffii</i> , 9003	98.2 (good)	0.69	return sludge, paper mill 1
<i>A. baumannii</i> , 910045	99.9 (excellent)	0.99	enrichment culture of activated sludge, pulp mill 1
<i>A. junii</i> , 910093	92.0 (good)	0.99	enrichment culture of activated sludge, pulp mill 1
<i>A. junii</i> , 920121	92.0 (good)	1.00	influent wastewater, pulp mill 2
<i>Agrobacter radiob.</i> , 920012	92.8 (good)	0.72	activated sludge, paper mill 1
<i>Pseudomonas</i> sp., 920146	a)		return sludge, pulp mill 2
Non-identifiable, 920137			activated sludge, pulp mill 2

a) The API 20 NE gives varying identification profiles for this isolate: *Pseudomonas stutzeri* 95.7 % (good) or *Pseudomonas vesicularis* 88.6 % (acceptable).

both the phosphate used for biomass growth and that stored as polyphosphates was included in the measured phosphate uptake.

### Phosphate uptake in short-term batch cultures

Three *Acinetobacter* isolates, *A. lwoffii* (isolate 9003), *A. baumannii* (isolate 910045) and *A. junii* (isolate 910093), and three other isolates *Agrobacter radiobacter* (isolate 920012), *Pseudomonas* sp. (isolate 920146) and a non-identifiable isolate 920137 were studied in short-term batch cultures (Table 6).

The bacterial biomass used in phosphate uptake experiments was grown as a batch culture on a phosphorus-limited acetate mineral medium to produce cells devoid of polyphosphate granules (checked with the Neisser stain). The concentrations of the main elements in this medium were as follows: 570.0 mg l<sup>-1</sup> C, 4.0 mg l<sup>-1</sup> P, 40.0 mg l<sup>-1</sup> N, 8.0 mg l<sup>-1</sup> Mg, 8.0 mg l<sup>-1</sup> K, 3.0 mg l<sup>-1</sup> Ca. The trace mineral solution was added as described in chapter 2.4. The medium was buffered to pH 7.0 with HEPES, 7 g l<sup>-1</sup> (pK<sub>a</sub> = 7.55).

These concentrations were selected on the basis of the preliminary results of growth optimization tests with a view that a fairly high biomass concentration would be obtained with a low phosphate concentration relative to carbon. It seems to be important to have a sufficient supply of energy (i.e. organic carbon) in a phosphorus-limited culture during the starvation period (Rosenberg et al. 1969). The phosphorus-limited batch cultures were incubated at 30 °C on a rotary shaker (120 rpm) overnight (17 – 21 h). It is important to incubate the bacteria under phosphorus limitation long enough to produce cells without any polyphosphates, but too long a starvation period should be avoided. In *Escherichia coli* prolonged phosphorus starvation has been observed to reduce the viability of cells, probably due to utilization of ribonucleic acid as a phosphorus source (Mallette et al. 1964).

For the phosphate uptake experiment the biomass was centrifuged (10 minutes, 8000 rpm, 10 °C, Sorvall Superspeed RC2-B centrifuge), washed with sterile 0.9 % saline and centrifuged again. The biomass was suspended in 30 – 60 ml of the phosphate uptake medium resulting in a dry mass of 1 – 4 mg l<sup>-1</sup>. The initial phosphate concentration of the uptake medium varied from

4.0 to 40.0 mg l<sup>-1</sup> P. The carbon concentration was 120.0 mg l<sup>-1</sup> C. Otherwise the composition of the medium was the same as that of the phosphorus-limited medium described above.

The low carbon concentration (120.0 mg l<sup>-1</sup> C) and the short incubation time were used in order to minimize bacterial growth and incorporation of phosphorus into cell material instead of polyphosphate accumulation.

The antibiotic streptomycin sulphate, which prevents growth but not polyphosphate formation, has been used in studies of excess phosphate uptake by van Groenestijn et al. (1988). Streptomycin sulphate may, however, stimulate phosphate uptake (van Groenestijn et al. 1988). It was not used in the present study, since the unknown stimulation of phosphate uptake might have caused a larger error than limited growth.

Phosphate uptake in batch cultures each having a different initial phosphate concentration was followed as a function of time. The cultures were incubated at 30 °C on a rotary shaker (120 – 130 rpm) for 0.5 – 1.0 h. However, *Pseudomonas* sp. and the non-identifiable isolate 920137 had to be incubated as long as 11 and 25 h, respectively, before their phosphate uptake rates could be calculated. Samples for phosphate analyses were filtered through an Acrodisc PF syringe filter with a 0.2 µm final filter (a more detailed description in chapter 2.2). The supernatant was analyzed colorimetrically for orthophosphate according to the standard SFS 3025.

Total colony counts were determined as colony forming units on the TGY (1:5) medium at the beginning of the uptake experiment and after 30 min. incubation. Bacterial dry mass (DM) was determined at the beginning of the experiment by centrifuging (10 min., 8000 rpm, 10 °C) the cells from the suspension, washing the biomass with sterile 0.9 % saline and drying the pellet according to the SFS standard 3008, or in some experiments by filtrating the sample through a 0.45 µm Millipore filter and drying the filter at 105 °C until constant weight was achieved.

Polyphosphate formation in the cells was followed with a microscope after dyeing with the Neisser stain (Gurr 1965).

At the end of the experiment the pH value was measured with an indicator paper (Merck).

The phosphate uptake rate for each initial phosphate concentration was calculated from the



linear range of the uptake curve (usually the first 15 to 30 minutes). The initial phosphate uptake rates of the three *Acinetobacter* isolates were pooled and plotted as a function of the corresponding initial phosphate concentration. These plots were analyzed for their kinetic characteristics. The data were fitted by the least squares method of Levenberg-Marquardt for non-linear functions (Guide to Standard Mathematica® Packages 1992) to the Michaelis-Menten equation (1):

$$v_o = \frac{V_{\max} \cdot P_o}{K_M + P_o} \quad (1)$$

where

$v_o$  = initial uptake rate of phosphate,  
mg P g<sup>-1</sup> DM h<sup>-1</sup>

$V_{\max}$  = maximum initial uptake rate of phosphate,  
which is being approached with increasing  
concentrations of P, mg P g<sup>-1</sup> DM h<sup>-1</sup>

$P_o$  = initial concentration of phosphate in the  
medium, mg l<sup>-1</sup> P

$K_M$  = the Michaelis constant or the half-  
saturation constant, i.e. the concentration  
of P where the initial uptake rate of  
phosphate is half of the maximum initial  
uptake rate, mg l<sup>-1</sup> P

### Phosphate uptake during growth in batch cultures

Phosphate uptake kinetics during growth was studied with pure cultures of four *Acinetobacter* isolates: *A. lwoffii*, isolate 9003, *A. baumannii*, isolate 910045 and *A. junii*, isolates 920093 and 920121 (Table 6).

The inoculum for the experiment was grown in the phosphorus-limited acetate mineral medium as described above for the short-term batch cultures. This culture was inoculated into the phosphate uptake medium to give a final concentration of 6 % of the inoculum in the medium (absorbance approximately 0.05 at 600 nm). The bacterial dry mass in the phosphate uptake medium at the beginning of the experiment was calculated on the basis of the dry mass of the inoculum suspension (SFS 3008) and the inoculation percentage.

As it was luxury uptake of phosphate under favourable growth conditions that was to be

studied (chapter 1.4.4), the phosphate uptake medium was composed so as to fulfil the optimal growth requirements of acinetobacters. The concentrations of carbon (570.0 mg l<sup>-1</sup> C), nitrogen (80.0 mg l<sup>-1</sup> N) and phosphorus (8.0 mg l<sup>-1</sup> P) in the medium were the same as those producing maximum growth in the three *Acinetobacter* isolates (chapter 3.5). The concentrations of potassium (10.0 mg l<sup>-1</sup> K) and magnesium (9.0 mg l<sup>-1</sup> Mg) were also selected on the basis of the growth optimization experiments, although the optimal concentrations of these elements varied a great deal, depending on the isolate. The concentration of calcium (7.0 mg l<sup>-1</sup> Ca) was based on the molar ratio of Mg:Ca = 2.1:1 in the recipe given by van Groenestijn et al. (1988). The trace mineral solution was added as described in chapter 2.4. The pH was adjusted with PIPES (pK<sub>a</sub> = 6.8) to a slightly acidic value (6.8), because pH tended to rise during bacterial growth in spite of good buffering of the medium.

The cultures were incubated at 30 °C on a rotary shaker (130 rpm) until orthophosphate was exhausted in the medium. The phosphate concentrations were usually determined after 0, 5, 7, 8, 9, 10, 12 h incubation periods and finally between 18 and 24 h, depending on the rate of phosphate uptake. Some experiments also covered the time between 12 and 18 h. Samples for the phosphate analyses were filtered and analyzed as described earlier. The pH was measured at the same sampling times as phosphate.

Bacterial growth was followed by measuring the optical density (600 nm, with a Hitachi U-2000 spectrophotometer and, to compare with the results of the anaerobic experiments, also through the side arm of the Erlenmeyer flask with a Spectronic 20 D spectrophotometer) usually after 0, 3, 5, 6, 7, 8, 9, 10, 11, 12 h incubation periods and between 18 and 24 h. The cell number was counted under the microscope (usually after 0, 5, 7, 9, 12, 18 – 24 h) after acridine orange staining (Hobbie et al. 1977) by using a Leitz SM Lux epifluorescent microscope and a magnification of 1250. At least 400 cells were counted for one sample. The bacterial dry mass was determined at the end of the experiment (SFS 3008).

Polyphosphate formation in the cells was followed under the microscope after dyeing with the Neisser stain (Gurr 1965).

The kinetic parameters were calculated by

fitting the data to equations (2) and (3)

$$\frac{dP}{dt} = -\frac{\mu_{\max} \cdot P}{W(K_s + P)} \cdot B \quad (2)$$

$$\frac{dB}{dt} = \frac{\mu_{\max} \cdot P}{(K_s + P)} \cdot B \quad (3)$$

where

P = orthophosphate concentration ( $\mu\text{g ml}^{-1}$  P)

t = time (h)

$\frac{dP}{dt}$  = uptake rate of phosphate ( $\mu\text{g ml}^{-1} \text{ h}^{-1}$  P)

B = bacterial biomass ( $10^7$  cells  $\text{ml}^{-1}$ )

$\frac{dB}{dt}$  = rate of biomass formation  
( $10^7$  cells  $\text{ml}^{-1} \text{ h}^{-1}$ )

$\mu_{\max}$  = maximum specific growth rate of bacteria ( $\text{h}^{-1}$ )

$K_s$  = half-saturation constant ( $\mu\text{g ml}^{-1}$  P), i.e. the orthophosphate concentration where the specific growth rate  $\mu = 1/2\mu_{\max}$

W = yield coefficient, i.e. the ratio for converting a phosphate unit into a biomass unit ( $10^7$  cells ( $\mu\text{g}^{-1}$  P)

The maximum uptake rate of phosphate ( $V_{\max}$ ,  $\mu\text{g P (}10^7 \text{ cells)}^{-1} \text{ h}^{-1}$ ) can be calculated with the equation  $V_{\max} = \frac{\mu_{\max}}{W}$ , and the specific growth rate ( $\text{h}^{-1}$ ) of bacteria with the equation  $\mu = \frac{1}{B} \cdot \frac{dB}{dt}$ .

Equations 2 and 3 have been derived from the Michaelis-Menten equation (Michaelis and Menten 1913) as presented by Curds and Bazin (1977). The equations relate the phosphate uptake rate and the biomass formation to phosphate and biomass concentrations. Time delays between phosphate uptake and bacterial growth were taken into consideration when fitting the data to the model.

The actual phosphate uptake rate and the maximum uptake rate per dry mass of bacteria can be calculated by using the following equation:  $DM = b \cdot B$ , where DM = dry mass (as  $\mu\text{g DM ml}^{-1}$ ), b is the coefficient relating dry mass to cell density (as  $\mu\text{g DM (}10^7 \text{ cells)}^{-1}$ ) and B

is the biomass expressed as cell density (as  $10^7$  cells  $\text{ml}^{-1}$ ).

### Phosphorus content of *Acinetobacter* biomass

The phosphorus content of bacterial biomass (a sample of 1.0 to 1.5 ml) was analyzed according to the standard method used for determination of total phosphorus in water (SFS 3026). The method is based on the decomposition of inorganic and organic phosphorus compounds to orthophosphate with peroxodisulphate under acidic conditions and a pressure of 200 kPa (corresponding to 120 °C).

The peroxodisulphate treatment is known to be efficient enough for determination of total phosphorus in most municipal wastewater samples. However, by way of comparison the total phosphorus concentrations of some samples of *Acinetobacter* biomass after a short-term phosphate uptake experiment were also analyzed according to a method for determining total phosphorus in wastewater. This method brings about a more efficient degradation of organic matter by heating peroxodisulphate with concentrated sulphuric acid (a modification of the Swedish standard SS 02 81 02). Since there were no significant differences in the results obtained with these two methods, the simpler peroxodisulphate method (SFS 3026) was chosen. This method has also been used e.g. by Hiraishi and Kitamura (1984) for analyzing the phosphorus content of polyphosphate-containing biomass.

Samples were taken before the cells were suspended in the phosphate uptake medium (i.e. the biomass devoid of polyphosphate storage) and at the end of the uptake experiment.

The total phosphorus content was also estimated on the basis of phosphate taken up from the medium. At the end of the short-term batch experiments phosphorus was not analyzed, but only estimated.

### 2.9 Phosphate release kinetics of five *Acinetobacter* isolates

Release of orthophosphate from the cells of *Acinetobacter lwoffii*, isolates 9003 and 920122, *A. baumannii*, isolate 910045, and *A. junii*, isolates

910093 and 920121 (for a description of the isolates except for isolate 920122, see Table 6) was studied by exposing aerobic cultures to anaerobic conditions. *A. lwoffii*, isolate 920122 (identification 95.9 %, good, T value 0.95) originated from the same influent wastewater sample from pulp mill 2 as *A. junii*, isolate 920121.

The anaerobic experiments were made both after the short-term batch experiments (with negligible growth) and after the experiments with growing cultures (after 20 – 24 h growth periods).

According to the literature, acidic conditions and carbon additions are needed for phosphate release (Fuhs and Chen 1975). This assumption was confirmed in the preliminary experiments with *A. baumannii*, isolate 910045. For this reason the phosphate release experiments were performed after adjusting pH in the culture to 6.0 and adding carbon as sodium acetate to achieve a final concentration of 330 mg l<sup>-1</sup> C. By way of comparison, some experiments were performed at pH 7.0.

The Erlenmeyer flasks with side arms (Bellco) were sealed with sterile, air tight rubber stoppers. Anaerobic conditions were created by purging the culture of all oxygen with N<sub>2</sub> for 5 min (the gas was sterilized with an Acrodisc syringe filter of 0.2 µm) and by adding sodium thioglycollate (E<sub>0</sub>' = 100 mV, Merck) as a reductant in a final concentration of 0.05 %. Resazurin (BDH Chemical Ltd Poole, England) in a final concentration of 0.0001 % was used as a redox indicator. Resazurin turns from red to colourless when E<sub>h</sub> is approximately -110 mV. Because resazurin is light-sensitive, the anaerobic culture flask was wrapped in black plastic.

The culture was incubated at 30 °C on a rotary shaker (130 rpm) usually for 24 h. The same parameters (orthophosphate, optical density, cell density, dry mass, pH) as in the aerobic uptake experiments were measured as a function of time (usually 2, 4, 6 and 24 h after the onset of anaerobiosis). During the experiments, samples for orthophosphate and pH were taken aseptically through the septum by using an Acrodisc PF syringe flushed with N<sub>2</sub>. The optical density was measured at 600 nm (Spectronic 20D, Milton Roy) through the side arm of the Erlenmeyer flask. At the end of the experiment, optical density was also measured by using a Hitachi

U-2000 spectrophotometer. Samples for cell density and dry mass measurements, as well as for Neisser staining were taken only at the end of the experiment.

The amount of phosphate released was calculated as a percentage of the increase in phosphate concentration during the anaerobic phase in relation to the decrease of phosphate concentration (i.e. phosphate bound) during the aerobic phase. No growth was observed during the anaerobic experiments.

Since phosphate uptake was linear with respect to time, the release rates were calculated by linear regression.

The phosphorus concentration of the biomass at the end of the anaerobic period was determined as described in chapter 2.8.

## 2.10 Denitrification potential of *Acinetobacter* isolates

The denitrification potential of all 57 *Acinetobacter* isolates was studied by recultivating 100 µl of the stock bacterial culture in 25 ml of a medium containing 8 g l<sup>-1</sup> nutrient broth (Difco) and nitrate (70 mg l<sup>-1</sup> N) on a rotary shaker (120 rpm) at 30 °C until early stationary phase. 1.0 ml of this cell suspension was inoculated into an anaerobic culture tube (Bellco) containing 10 ml of the nutrient broth and nitrate medium and sealed with a butyl rubber stopper.

Before autoclavation, the headspace of the culture tube was flushed with nitrogen gas (N<sub>2</sub>), and before inoculation, 1.5 ml of acetylene was added through a sterile 0.22 µm filter. Acetylene inhibits the conversion of nitrous oxide (N<sub>2</sub>O) to N<sub>2</sub>, and thus the produced N<sub>2</sub>O can be taken as an estimate of the total amount of gas produced (N<sub>2</sub> + N<sub>2</sub>O).

The tubes were incubated without stirring at 30 °C for 6 d. The optical densities of the cultures were measured after 2 and 6 d with a spectrophotometer (Spectronic 20D, Milton Roy). After six days the tubes were shaken vigorously to distribute the produced N<sub>2</sub>O in equilibrium between the water and gas phase. A gas sample of 0.3 ml was withdrawn by a syringe and injected into a 10 ml evacuated blood collecting tube (Venoject, Terumo Europe, Belgium). The remaining volume of the Venoject tube was filled with N<sub>2</sub>. The gas samples were

stored for later analysis of  $N_2O$ . The disappearance of nitrate in the liquid medium was tested in a 0.5 ml sample by dropwise addition of a diphenylamine solution (0.2 g  $(C_6H_5)_2NH$  in 100 ml of concentrated sulphuric acid). Blue colour indicates the presence of nitrate or nitrite, and a colourless reaction is presumptive evidence of denitrification (Tiedje 1982).

Analysis of  $N_2O$  was performed by gaschromatography with a Hewlett-Packard 8690 gaschromatograph equipped with an electron capture detector. Separation was carried out on a 2 m long Porapak Q column at 50 °C by using  $ArCH_4$  as the carrier gas (30 ml min<sup>-1</sup>).

Sterile control tubes were prepared and handled in the same manner as the inoculated tubes. A denitrifying *Pseudomonas* isolate (obtained from K. S. Jørgensen) was used as a control sample for total conversion of nitrate to gas.

Bacteria converting more than 80 % of nitrate to  $N_2O$  in the presence of acetylene are considered as true denitrifiers (Tiedje 1988).

### 3 RESULTS

#### 3.1 Volatile fatty acids and dissolved organic carbon in activated sludge treatment plants

In the influent to the treatment plant of pulp mill 1 the concentrations of volatile fatty acids were low (10 – 20 mg l<sup>-1</sup> C, Table 7), being only about 5 % of the DOC in the same samples (Table 8). In the influents to the treatment plants of the

Table 7. Concentrations of volatile fatty acids in the filtrates of activated sludge, in the influent to the treatment plants of pulp and paper mills and in the effluent.

Treatment plant	Concentration (mg l <sup>-1</sup> C)				
	Formic acid (n) <sup>a)</sup>	Acetic acid (n)	Propionic acid (n)	Butyric acid (n)	All acids
<b>Pulp mill 1</b>					
Influent <sup>b)</sup>	6.4 – 6.9 (3)	8.0 – 14.0 (4)	1.4 (1)	< 2.7 (1)	10 – 20
BA <sup>c)</sup>	0 – 0.3 (3)	0 – 0.7 (4)	na <sup>d)</sup>	na	0 – 1.0
EA <sup>e)</sup>	0.3 – 0.6 (3)	0 – 1.7 (4)	na	na	0.3 – 2.3
RS <sup>f)</sup>	0 – 0.5 (3)	0 – 0.7 (4)	na	na	0 – 1.2
<b>Paper mill 1</b>					
Influent	2.8 – 5.6 (2)	80.4 – 114.4 (2)	18.5 – 31.6 (2)	12.5 – 25.1 (2)	117 – 174
BA	2.1 (1)	2.5 (1)	0 (1)	0 (1)	4.6
Effluent <sup>g)</sup>	1.1 (2)	0.96 – 1.1 (2)	0 (2)	0 (2)	2
<b>Paper mill 2</b>					
EB <sup>h)</sup>	0.5 (1)	52.8 (1)	8.3 (1)	2.1 (1)	63.7
Influent	2.2 (1)	52.0 (1)	3.0 (1)	1.7 (1)	58.9
RS	0.4 (1)	0.8 (1)	0.1 (1)	0 (1)	1.3
<b>Paper mill 3</b>					
PS <sup>i)</sup>	3.1 (1)	29.4 (1)	8.1 (1)	10.5 (1)	51.1
EB	3.2 (1)	52.8 (1)	14.0 (1)	26.4 (1)	96.4
BA	1.4 (1)	1.4 (1)	0 (1)	0 (1)	2.8

a) n = number of samples analyzed

b) Influent = wastewater from the equalization basin to the aeration basin

c) BA = beginning of the aeration basin

d) na = not analyzed

e) EA = end of the aeration basin

f) RS = return sludge

g) Effluent = purified wastewater to the recipient

h) EB = equalization basin

i) PS = wastewater after presedimentation = influent to the equalization basin

paper mills the concentrations were higher (60 – 174 mg l<sup>-1</sup> C), being 30 – 40 % of the DOC. The volatile fatty acids were assimilated immediately in the aeration basin: the concentrations were almost zero already at the beginning of the aeration phase.

In the samples from the treatment plant of pulp mill 2 only the DOC concentrations were determined. These concentrations were also considerably reduced at the beginning of aeration, but no marked changes occurred at the later stages when the wastewater passed through the other parts of the treatment plant. Half of the DOC was not degraded in the aeration basin.

In the treatment plants of the paper mills 15 – 25 % of the DOC was not degraded in the aeration basin.

Table 8. Concentrations of dissolved organic carbon (DOC) in the filtrates of activated sludge and in the influents to the treatment plants of pulp and paper mills.

Treatment plant	DOC (mg l <sup>-1</sup> C) (n) <sup>a)</sup>
<b>Pulp mill 1</b>	
Influent <sup>b)</sup>	194 – 386 (3)
<b>Pulp mill 2</b>	
Influent	323 (1)
BA <sup>c)</sup>	161 (1)
RS <sup>d)</sup>	160 (1)
<b>Paper mill 1</b>	
Influent	312 – 393 (2)
BA	49 – 72 (2)
Return sludge	45 – 63 (2)
<b>Paper mill 2</b>	
Influent	158 – 169 (4)
EA <sup>e)</sup>	42 (1)
RS	34 – 39 (3)
<b>Paper mill 3</b>	
EB <sup>f)</sup>	226 (1)
RS	61 (1)

a) n = number of samples analyzed

b) Influent = wastewater from the equalization basin to the aeration basin

c) BA = beginning of the aeration basin

d) RS = return sludge

e) EA = end of the aeration basin

f) EB = equalization basin

### 3.2 Potassium, magnesium and calcium in activated sludge treatment plants

The concentrations of potassium (K), magnesium (Mg) and calcium (Ca) varied little in the different parts of a particular treatment plant (Table 9). The temporal variations were small as well. Every treatment plant seemed to have a concentration level of these elements that was characteristic of it. In the treatment plants of the pulp mills and of paper mill 1, Mg concentrations varied between 4.4 and 9.0 mg l<sup>-1</sup> Mg, and of paper mills 2 and 3 between 2.5 and 4.0 mg l<sup>-1</sup> Mg. Concentrations of Ca were always high. The treatment plant of paper mill 1 had by far the highest K concentrations (13 – 28 mg l<sup>-1</sup> K).

### 3.3 Acinetobacters and other aerobic heterotrophic bacteria in wastewater and activated sludge

Only 15 % of the colonies isolated on the acetate mineral medium were identified as acinetobacters. The majority of the bacteria (59 %, Tables 10 and 11) could not be identified with the API 20 NE identification system. Most of the identifiable isolates were identified as *Pseudomonas*, *Pasteurella* and *Chryseomonas*.

In most samples (62 %) there were no acinetobacters among the colonies identified. This does not, however, mean that these bacteria would not have been present. For instance, with 10 colonies identified and with a A/TGY ratio (the ratio of the number of colonies on the acetate mineral medium, A, to the colonies on the TGY medium) of 20 % only acinetobacter frequencies above 2 % of the total count could be observed.

The total number of aerobic heterotrophic bacteria can be calculated on the basis of the figures in Table 10 by using information given on the colony forming units (CFU) on the acetate mineral medium (A) and the ratio A/TGY. Usually the numbers of colony forming units on the TGY medium were not significantly higher after 5 – 7 days of incubation than after 2 – 3 days of incubation.

In the enrichment cultures 32 % of all the colonies identified were acinetobacters, which is clearly a higher percentage than in the non-enriched samples. Non-identifiable bacteria and

Table 9. Concentrations of potassium (K), magnesium (Mg) and calcium (Ca) in the filtrates of activated sludge, in the influents (after neutralization) to the treatment plants of pulp and paper mills and in the effluent.

Treatment plant	Concentration (mg l <sup>-1</sup> )		
	K (n) <sup>a)</sup>	Mg (n)	Ca (n)
<b>Pulp mill 1</b>			
Influent <sup>b)</sup>	8.1 – 11 (4)	4.8 – 6.6 (4)	132 – 225 (4)
BA <sup>c)</sup>	7.6 – 11 (4)	4.6 – 6.6 (4)	120 – 210 (4)
EA <sup>d)</sup>	7.6 – 12 (4)	4.4 – 7.0 (4)	112 – 210 (4)
RS <sup>e)</sup>	8.4 – 11 (4)	4.8 – 7.0 (4)	124 – 210 (4)
Effluent <sup>f)</sup>	8.4 – 9.6 (2)	4.8 – 6.4 (2)	128 – 205 (2)
<b>Pulp mill 2</b>			
Influent	9.3 (1)	7.9 (1)	129 (1)
BA	8.5 (1)	7.1 (1)	119 (1)
RS	9.2 (1)	7.3 (1)	118 (1)
<b>Paper mill 1</b>			
Influent	20 – 28 (9)	6.0 – 9.0 (9)	34 – 280 (9)
BA	13 – 19 (2)	7.1 – 8.0 (2)	100 – 310 (2)
EA	17 (1)	7.0 (1)	100 (1)
RS	14 – 20 (2)	7.5 – 8.2 (2)	110 – 320 (2)
<b>Paper mill 2</b>			
Influent	6.3 – 10 (4)	2.5 – 3.1 (4)	67 – 120 (4)
BA	10 (1)	3.5 (1)	110 (1)
EA	9.7 (1)	3.5 (1)	110 (1)
RS	8.0 – 11 (3)	3.2 – 3.5 (3)	77 – 110 (3)
<b>Paper mill 3</b>			
EB <sup>g)</sup>	17 – 18 (2)	3.8 – 4.0 (2)	40 – 220 (2)

a) n = number of samples analyzed

b) Influent = wastewater from the equalization basin to the aeration basin

c) BA = beginning of the aeration basin

d) EA = end of the aeration basin

e) RS = return sludge

f) Effluent = purified wastewater to the recipient

g) EB = equalization basin

Table 10. Number of cultivable bacteria isolated from different parts of the treatment plants of pulp and paper mills.

Treatment plant and date	Ac./tot. <sup>a)</sup>	Identification of isolates (n) <sup>b)</sup>	Acetate medium				A/TGY <sup>e)</sup>	
			10 <sup>6</sup> CFU ml <sup>-1</sup> <sup>c)</sup>		10 <sup>9</sup> CFU g <sup>-1</sup> dry sludge <sup>d)</sup>		%	
			2-3d	5-7d	2-3d	5-7d	2-3d	5-7d
<b>Pulp mill 2</b>								
11.3.1992 INF <sup>h)</sup>	0/10		0	0.20±0.06	0	0.05	0	13
		<i>Ps.</i> <sup>g)</sup> <i>paucimobilis</i> (1) <i>Pasteurella</i> sp. (1) NI <sup>h)</sup> (8)						
BA <sup>i)</sup>	0/10		1.5±0.5	130±20	0.25	22	18	33
		<i>Ps. paucimobilis</i> (1) <i>Ps. vesicularis</i> (1) <i>Ps. cepacia</i> (1) <i>Flavobacterium</i> sp. (1) NI (6)						
RS <sup>j)</sup>	0/10		3.8±0.8	76±12	0.43	8.6	32	19
		NI (10)						
24.4.1992 INF	10/10		0.13±0.02	0.21±0.02	0.05	0.09	52	84
		<i>A. junii</i> (9) <i>A. lwoffii</i> (1)						
BA	0/10		6.5±3.6	52±10	1.0	8.1	7	17
		<i>Ps. vesicularis</i> (3) <i>Ps. mesophilica</i> (1) NI (6)						
RS	0/10		14±5	92±13	1.4	9.3	9	16
		<i>Pseudomonas</i> sp. (2) <i>Ps. paucimobilis</i> (1) NI (7)						
<b>Paper mill 1</b>								
24.4.1991 PSE <sup>k)</sup>	0/20		nd <sup>l)</sup>	0.65	nd	nd	nd	nd
		<i>Chryseomonas luteola</i> (9) NI (11)						
RS	1/9		nd	nd	nd	nd	nd	nd
		<i>Acinetobacter</i> sp. (1) <i>Ps. vesicularis</i> (2) <i>Ps. paucimobilis</i> (1) <i>Chryseomonas luteola</i> (1) NI (5)						
4.6.1991 RS	0/7		nd	36±7	nd	3.4	nd	nd
		NI (7)						
29.1.1992 PSE	1/10		0.47±0.08	1.1±0.1	nd	nd	10	24
		<i>Acinetobacter baumannii</i> (1) <i>Chryseomonas luteola</i> (2) NI (7)						
EA <sup>m)</sup>	0/10		nd	160±20	nd	15	nd	30
		<i>Pseudomonas paucimobilis</i> (2) <i>Agrobacter radiobacter</i> (2) NI (6)						
RS	0/10		70±12	240±20	4.4	15	11	17
		<i>Pasteurella</i> sp. (3) <i>Ps. paucimobilis</i> (1) <i>Ps. vesicularis</i> (1) <i>Flavobacterium indologenes</i> (1) NI (4)						

Treatment plant and date	Ac./tot. <sup>a)</sup>	Identification of isolates (n) <sup>b)</sup>	Acetate medium				A/TGY <sup>c)</sup>	
			10 <sup>6</sup> CFU ml <sup>-1</sup> <sup>c)</sup>		10 <sup>9</sup> CFU g <sup>-1</sup> dry sludge <sup>d)</sup>		%	
			2-3d	5-7d	2-3d	5-7d	2-3d	5-7d
31.3.1992 PSE	0/10	<i>Ps. cepacia</i> (1) <i>Flavobacterium odoratum</i> (1) NI (8)	0.50±0.09	2.0±0.6	0.22	0.88	18	56
BA	0/10	<i>Ps. paucimobilis</i> (2) <i>Agrobacter radiobacter</i> (2) NI (6)	0.95±0.42	15±17	0.07	1.1	1	3
RS	0/10	<i>Ps. paucimobilis</i> (2) NI (8)	0	36±26	0	2.5	0	7
<b>Paper mill 2</b> 3.10.1991 INF	4/10	<i>Acinetobacter</i> sp. (4) <i>Ps. putrefaciens</i> (1) <i>Pseudomonas</i> sp. (1) <i>Chryseomonas luteola</i> (1) NI (3)	4.0±2.7	16±0.5	nd	nd	13	70
RS	5/10	<i>Acinetobacter</i> sp. (5) NI (5)	18±6	50±10	1.4	4.0	15	28
31.10.1991 INF	0/10	<i>Chryseomonas luteola</i> (4) <i>Pasteurella</i> sp. (2) NI (4)	10±5	14±5	8.4	12	67	88
RS	2/10	<i>Acinetobacter</i> sp. (2) <i>Pasteurella</i> sp. (1) NI (7)	13±5	45±9	1.2	4.3	15	38
26.2.1992 INF	2/10	<i>Acinetobacter</i> sp. (2) <i>Ps. paucimobilis</i> (2) <i>Chryseomonas luteola</i> (1) <i>Oligella urethralis</i> (1) <i>Flavobacterium</i> sp. (1) NI (3)	0.82±0.39	8.4±1.3	0.68	0.83	1	14
EA	0/10	<i>Ps. paucimobilis</i> (1) NI (9)	0.05±0.01	20±6	0.009	3.7	0.5	26
RS	0/10	<i>Ps. paucimobilis</i> (1) <i>Flavobacterium breve</i> (1) NI (8)	0.05±0.10	26±5	0.009	4.9	0.2	20
<b>Paper mill 3</b> 21.5.1991 EB <sup>e)</sup>	3/18	<i>Acinetobacter</i> sp. (3) <i>Pasteurella</i> sp. (3) <i>Ps. vesicularis</i> (1) <i>Agrobacter radiobacter</i> (1) NI (10)	24±6.9	390±88	nd	nd	nd	nd



Treatment plant and date	Ac./tot. <sup>a)</sup>	Identification of isolates (n) <sup>b)</sup>	Acetate medium				A/TGY <sup>e)</sup>	
			10 <sup>6</sup> CFU ml <sup>-1</sup> <sup>c)</sup>		10 <sup>9</sup> CFU g <sup>-1</sup> dry sludge <sup>d)</sup>		%	
			2–3d	5–7d	2–3d	5–7d	2–3d	5–7d
RS	1/14	<i>Acinetobacter</i> sp. (1) <i>Ps. paucimobilis</i> (3) <i>Chryseomonas luteola</i> (1) <i>Pasteurella</i> sp. (1) NI (8)	22±7	nr <sup>o)</sup>	nd	nd	nd	nd
6.11.1991 EB	0/10	NI (10)	nd	20±2	nd		nd	99
RS	1/10	<i>Acinetobacter</i> sp. (1) <i>Pasteurella</i> sp. (1) NI (8)	nd	170±18	nd	nd	nd	17

- a) Ac./tot. = number of colonies identified as acinetobacters and total number of colonies identified in the sample  
b) n = number of isolates  
c) Number of colony forming units, CFU ml<sup>-1</sup> (after 2-3 d and 5-7 d incubation) on the acetate mineral medium with 95 % confidence limits  
d) CFU ml<sup>-1</sup> converted to 10<sup>9</sup> CFU g<sup>-1</sup> dry sludge  
e) A/TGY (%) = ratio of colonies on the acetate mineral medium to colonies on the TGY (1:5) medium  
f) INF = influent to the treatment plant  
g) *Ps.* = *Pseudomonas*  
h) NI = not identifiable with the API 20NE identification kit  
i) BA = beginning of the aeration basin  
j) RS = return sludge  
k) PSE = primary sedimentation basin  
l) nd = not determined  
m) EA = end of the aeration basin  
n) EB = equalization basin  
o) nr = result not reliable

*Pseudomonas* sp. were also common in the enrichment cultures (38 % and 24 %, respectively, of all colonies identified), especially in the influent.

The bacterial composition on the acetate mineral medium was basically very similar in the influent wastewater, activated sludge and enrichment cultures. Non-identifiable bacteria and *Pseudomonas* species were the most common (cf. Tables 10 and 11). However, in the enrichment cultures isolates identified as *Acinetobacter baumannii* were more common than in the sludge or wastewater samples.

#### Pulp mill 1

Bacteria in the treatment plant of pulp mill 1 had been studied earlier by Puustinen and Jørgensen

(unpublished results). They found that the number of colony forming units on the acetate mineral medium was on an average 5 % of the total number of cultivable bacteria on the TGY medium. None of the 21 colonies isolated was identified as *Acinetobacter*.

In the present study enrichment cultures were obtained from sludge of this treatment plant. In these cultures 0 – 86 % of the colonies identified were acinetobacters. The highest percentages were obtained in the sludge at the beginning of the aeration basin.

#### Pulp mill 2

In March 1992, when no nitrogen was added, about 10 % of the total number of cultivable bacteria in the influent grew on the acetate

Table 11. Bacteria isolated in the enrichment cultures of samples taken from different parts of the treatment plants of a pulp mill and a paper mill. In both cases data of two different samplings have been pooled.

Origin of the sample	Ac./tot. <sup>a)</sup>	Identification of isolates (n) <sup>b)</sup>
<b>Pulp mill 1</b>		
INF <sup>c)</sup>	4/26	<i>Acinetobacter lwoffii</i> (3) <i>Acinetobacter baumannii</i> (1) <i>Pseudomonas</i> sp. (1) <i>Ps.</i> <sup>d)</sup> <i>fluorescens</i> (1) <i>Ps. paucimobilis</i> (1) <i>Achrini</i> , CDC gr.VD (1) NI <sup>e)</sup> (18)
BA <sup>f)</sup>	14/17	<i>Acinetobacter junii</i> (6) <i>Acinetobacter baumannii</i> (5) <i>Acinetobacter</i> sp. (3) <i>Ps. aeruginosa</i> (1) <i>Alcaligenes faecalis</i> (1) NI (1)
EA <sup>g)</sup>	2/16	<i>Acinetobacter baumannii</i> (1) <i>Acinetobacter lwoffii</i> (1) <i>Pseudomonas</i> sp. (1) <i>Ps. putida</i> (2) <i>Ps. vesicularis</i> (1) <i>Ps. paucimobilis</i> (1) <i>Flavobacterium</i> sp. (1) <i>Comamonas testosteroni</i> / <i>Ps. alcaligenes</i> (2) NI (6)
RS <sup>h)</sup>	3/13	<i>Acinetobacter baumannii</i> (1) <i>Acinetobacter lwoffii</i> (1) <i>Acinetobacter</i> sp. (1) <i>Pseudomonas</i> sp. (3) <i>Ps. paucimobilis</i> (2) <i>Ps. stutzeri</i> (2) <i>Ps. aeruginosa</i> (1) NI (2)
<b>Paper mill 1</b>		
PSE <sup>i)</sup>	3/10	<i>Acinetobacter baumannii</i> (2) <i>Acinetobacter</i> sp. (1) <i>Ps. mesophilica</i> (1) NI (6)
RS	1/12	<i>Acinetobacter baumannii</i> (1) <i>Ps. paucimobilis</i> (1) <i>Ps. stutzeri</i> (1) NI (9)

a) Ac./tot. = number of colonies identified as acinetobacters and total number of colonies identified in the sample

b) n = number of isolates

c) INF = influent to the treatment plant

d) *Ps.* = *Pseudomonas*

e) NI = not identifiable with the API 20NE identification kit

f) BA = beginning of the aeration basin

g) EA = end of the aeration basin

h) RS = return sludge

i) PSE = primary sedimentation basin

mineral medium, whereas in the sludge the corresponding figure was 20 – 30 %. None of the colonies was identified as *Acinetobacter*, and as many as 80 % of these bacteria could not be identified. Almost all identifiable isolates belonged to the genus *Pseudomonas*. Acinetobacters constituted at the most 2 % of the total cultivable population of the aerobic heterotrophic bacteria. The pH value of the influent was 4.6.

A month later nitrogen was added to the treatment process. The pH value of the influent was 5.7. Half of the total number of the influent bacteria grew on the acetate mineral medium. All of the 10 colonies isolated were identified as acinetobacters (nine of them *A. junii* and one *A. lwoffii*). Thus, with the influent wastewater the treatment plant received an acinetobacter inoculum of  $1 \cdot 10^5$  CFU ml<sup>-1</sup>. About 10 % of the total number of cultivable bacteria in sludge grew on the acetate mineral medium, but there were no acinetobacters among the colonies identified. 65 % of the colonies were non-identifiable and 35 % belonged to the genus *Pseudomonas*.

#### Paper mill 1

In the influent wastewater after the primary sedimentation (PSE, i.e. unneutralized wastewater before the nutrient addition), the number of bacteria growing on the acetate mineral medium was 10 to 18 % of the total number on the TGY medium. Only 0 – 11 % of the sludge bacteria grew on the acetate mineral medium, and none of the isolates was *Acinetobacter*. *Chryseomonas luteola* was unusually common (45 % of the isolates) in the influent (PSE) sampled in April 1991. Otherwise *Pseudomonas* and non-identifiable bacteria were dominant on the acetate mineral medium in this treatment plant as well. Assuming that 10 % of the bacteria growing on the acetate mineral medium in 2 – 3 days were acinetobacters, the proportion of acinetobacters of the total number of cultivable aerobic heterotrophic bacteria would be less than 2 %.

In the enrichment cultures of sludge, 18 % of the colonies were identified as acinetobacters. All

of them were isolated in the enrichment obtained from the first sludge sample. Non-identifiable bacteria were the most frequent isolates (68 %).

### Paper mill 2

In early October 1991, almost half of the isolates on the acetate mineral medium were acinetobacters, corresponding to about 6 % of the total number of cultivable bacteria, whereas at the end of the month only 10 % of the isolates were acinetobacters. The nitrogen addition had been reduced by more than 50 % in the middle of the month. In February 1992, when the treatment plant had been operated with low nitrogen additions for a few months, the bacteria grew more slowly than usual on both acetate and TGY media. Especially on the acetate mineral medium the colonies were very small and often lost viability during the purification process. Apparently the bacterial population had changed, and those growing on the acetate mineral medium were not properly sustained by this medium. However, the total number of cultivable bacteria had not changed. The percentages of bacteria growing on the acetate mineral medium were much lower than earlier (1 % or below).

### Paper mill 3

In the the influent wastewater sample taken in November 1991, the number of colony forming units on the acetate mineral medium was the same as on the TGY medium. All of the colonies isolated on the acetate mineral medium were non-identifiable bacteria. The counts on the acetate mineral medium were generally higher in the treatment plant of paper mill 3 than in the other treatment plants. In May the high count on the acetate mineral medium of the influent samples was due to a great number of small colonies on the plates. 17 % of the bacteria isolated were acinetobacters. Assuming that the number of colony forming units on the acetate mineral medium was 20 % of the total count (which was not determined in this case), acinetobacters would have formed about 3 % of the total cultivable bacterial population in the influent and 1 % in the return sludge. Non-identifiable bacteria constituted the majority of bacteria in all samples

(69 % of all the isolates). Moreover, bacteria identified as *Pasteurella* sp. (10 %) and *Pseudomonas* sp. (8 %) occurred fairly frequently.

### 3.4 Optimal growth conditions for three *Acinetobacter* isolates

In the first factorial experiment performed with *Acinetobacter baumannii*, isolate 910045, only the concentration of carbon had a statistically significant effect on growth, i.e. carbon was the growth-limiting factor. However, the concentration of nitrogen apparently had a minor effect as well (Fig. 8).

On the basis of the first experiment the following regression model (equation 4, with the coded values) was obtained, describing the dependence of biomass formation (B, biomass in the stationary phase of growth expressed as absorbance · 100 at 600 nm) on carbon concentration (C)

$$B = 75.0 + 28.0 \cdot C - 9.69 \cdot C^2, \quad (4)$$

$$p = \leq 0.07, R^2 = 98.4 \%$$

Abbreviation p is the probability level for separate coefficients.  $R^2$  is the coefficient of

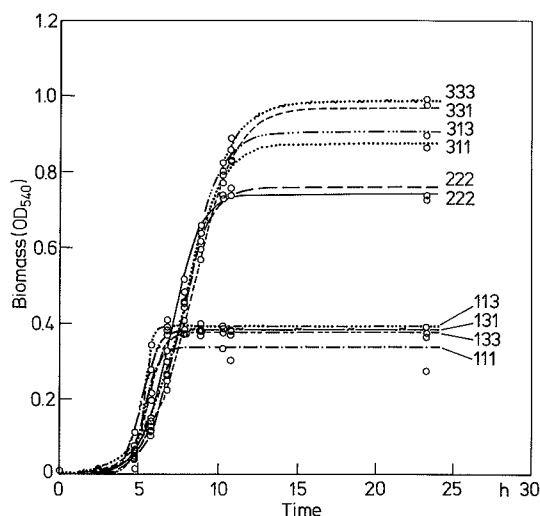


Fig. 8. Growth of *Acinetobacter baumannii*, isolate 910045, with varying carbon (C), nitrogen (N) and phosphorus (P) concentrations. Biomass at the stationary phase of growth is presented as optical density units (540 nm). Codes 333, 331 etc. mark the levels of C, N and P additions (in this order) with 3 being the highest and 1 the lowest concentration of C, N or P.

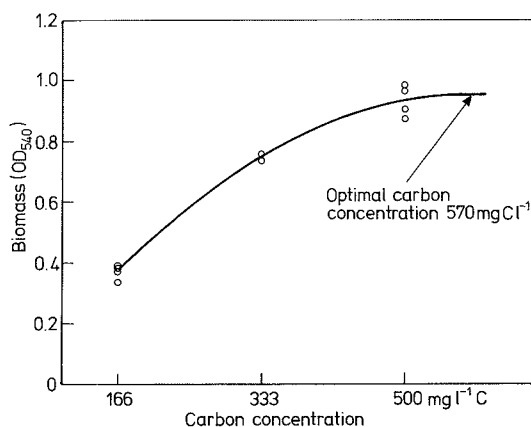


Fig. 9. Growth of *Acinetobacter baumannii*, isolate 910045, as a function of carbon concentration. Biomass at the stationary phase of growth is presented as optical density units (540 nm). The optimal carbon concentration was obtained by derivation.

determination expressing the percentage of variation in data explained by the regression equation.

Derivation of equation (4) gives the carbon concentration (the coded value) not limiting the growth of *A. baumannii* in phosphorus and nitrogen concentrations up to 30 mg l<sup>-1</sup> P and 140 mg l<sup>-1</sup> N. The coded optimal carbon concentration corresponds to a concentration of 570 mg l<sup>-1</sup> C (Fig. 9), which is somewhat higher than the highest concentration in the actual experiment. This concentration was used in the optimization experiments 2, 3 and 4. It was assumed that the other isolates would have the same response to carbon as *A. baumannii*.

From a methodological point of view it was important to use a carbon concentration where the bacteria could grow as a fairly thick suspension, as this increases the reliability of optical density measurements. With the carbon concentration of 570 mg l<sup>-1</sup> C and optimal concentrations of the other variables a suspension with an absorbance of about 0.9 (600 nm) was obtained. The lowest absorbances, which were measured with the unfavourable combinations of the variables, were about 0.2 (600 nm).

On the basis of experiments 2–4 the following regression models (equations 5–8, with the coded values) were obtained, describing the dependence of biomass formation (B, the biomass at the stationary phase of growth expressed as

absorbance · 100 at 600 nm) on the phosphorus (P), nitrogen (N), magnesium (Mg) and potassium (K) concentrations

for *Acinetobacter lwoffii* (9003)

$$B = 45.2 + 4.13 \cdot P + 2.99 \cdot K \cdot Mg - 4.91 \cdot P^2 - 3.66 \cdot N^2 - 3.76 \cdot Mg^2 - 2.74 \cdot K^2, \quad (5)$$

$p \leq 0.003, R^2 = 78.8 \%$

for *Acinetobacter baumannii* (910045)

$$B = 83.6 - 3.29 \cdot P + 4.57 \cdot N + 4.33 \cdot P \cdot N - 4.58 \cdot P^2 - 5.75 \cdot N^2 + 3.92 \cdot P^3 + 1.52 \cdot N^3, \quad (6)$$

$p \leq 0.001, R^2 = 96.8 \%$

for *Acinetobacter junii* (910093)

$$B = 6.21 + 1.85 \cdot P + 1.65 \cdot P \cdot N - 3.09 \cdot P^2 - 2.56 \cdot N^2 + 2.19 \cdot P^3 + 1.88 \cdot N^3, \quad (7)$$

$p \leq 0.004, R^2 = 91.5 \%$

$$B = 68.5 + 1.73 \cdot K + 2.75 \cdot Mg - 1.12 \cdot Mg^2, \quad (8)$$

$p \leq 0.07, R^2 = 89.3 \%$

Derivation of equations (5)–(8) gives the coded optimal concentrations, which can be calculated as mg l<sup>-1</sup> with the aid of the centre point and the step of the particular experiment (chapter 2.7).

The corresponding response contours are presented in Fig. 10a–c and Fig. 11a–b. The nitrogen-phosphorus models of *A. baumannii* and *A. junii* contain terms of third order, which give contours falling outside the experimental region (absorbances above 0.9 for *A. baumannii* and 0.7 for *A. junii*, Fig. 10b and c, respectively). In the case of the third order function the optimal concentration has been determined on the basis of the turning point.

Good growth in the three *Acinetobacter* isolates was obtained with a fairly wide range of nitrogen and phosphorus concentrations, as indicated by the large area inside the response contours of the highest absorbances (Fig. 10a–c). The optimal phosphorus concentrations were almost the same for all *Acinetobacter* isolates studied, i.e. on an average 8.3 mg l<sup>-1</sup> P (Table 12, Fig. 10a–c). The optimal nitrogen concentrations varied to some extent, the average being 79 mg l<sup>-1</sup> N. The optimal nitrogen concentration for *A. lwoffii* was at the centre point (65 mg l<sup>-1</sup> N) of the experimental region, when the magnesium and potassium concentrations were fixed at their

optimal levels of  $6 \text{ mg l}^{-1} \text{ Mg}$  and  $6 \text{ mg l}^{-1} \text{ K}$ .

The optimum C:N:P was 100:14:1.5 on an average. The optimum C:P ratio was 67, the optimum C:N ratio varied from 6 to 9 and the optimum N:P ratio from 8 to 12.

Responses to the magnesium concentrations varied. *A. lwoffii* reached maximum growth in the

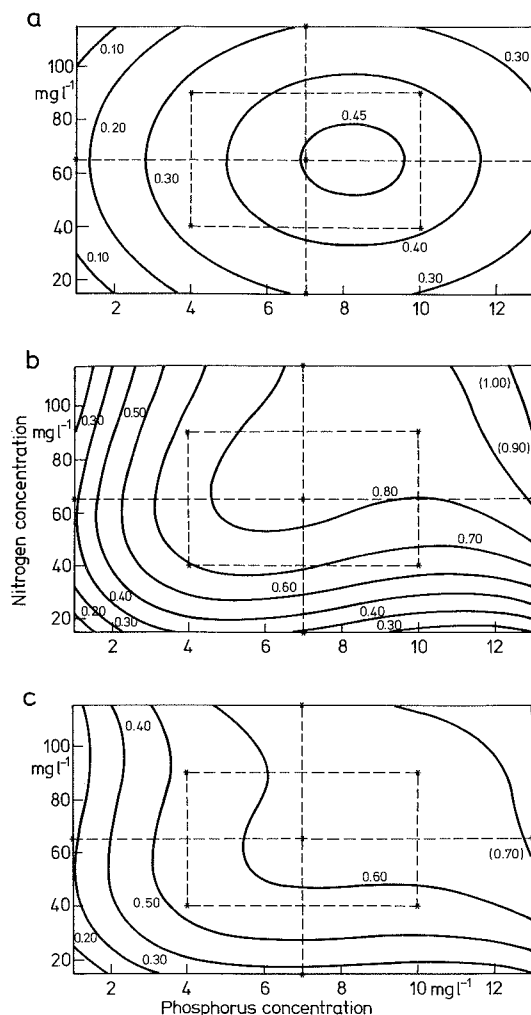


Fig. 10a - c. The effect of nitrogen and phosphorus on the growth of (a) *Acinetobacter lwoffii*, isolate 9003, (magnesium and potassium concentrations fixed at their optimal levels of  $6 \text{ mg l}^{-1}$  and  $6 \text{ mg l}^{-1}$ ), (b) *Acinetobacter baumannii*, isolate 910045, (contours 0.9 and 1.0 fall outside the experimental region and are given by the model), and (c) *Acinetobacter junii*, isolate 910093, (contour 0.7 falls outside the experimental region and is given by the model). The response contours show the biomass at the stationary phase of growth (presented as optical density units at 600 nm). The experimental region is presented with dotted lines.

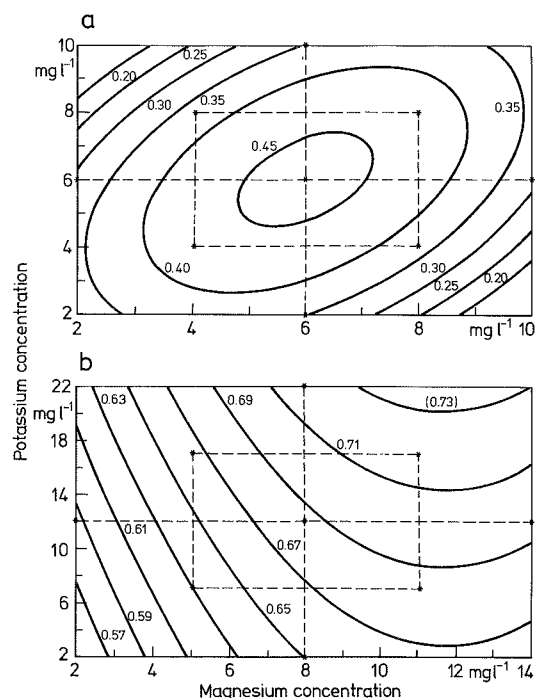


Fig. 11a - b. The effect of magnesium and potassium on the growth of (a) *Acinetobacter lwoffii*, isolate 9003, (nitrogen and phosphorus concentrations fixed at their optimal levels of  $65 \text{ mg l}^{-1}$  and  $8.3 \text{ mg l}^{-1}$ ) and (b) *Acinetobacter junii*, isolate 910093, (contour 0.73 falls outside the experimental region and is given by the model). The response contours show the biomass at the stationary phase of growth (presented as optical density units at 600 nm). The experimental region is presented with dotted lines. Note the difference in scales.

centre point of the experimental region at  $6.0 \text{ mg l}^{-1} \text{ Mg}$  (nitrogen and phosphorus concentrations at their optimal levels of  $65 \text{ mg l}^{-1} \text{ N}$  and  $8.3 \text{ mg l}^{-1} \text{ P}$ , Fig. 11a) and *A. junii* at  $11.7 \text{ mg l}^{-1} \text{ Mg}$  (Fig. 11b). *A. baumannii* showed no response to magnesium. The molar ratios of Mg:P were 0.93 and 1.8 for *A. lwoffii* and *A. junii*, respectively.

Responses to potassium concentrations varied as well. *A. lwoffii* had maximum growth at  $6.0 \text{ mg l}^{-1} \text{ K}$ , i.e. in the centre point of the experimental region (nitrogen and phosphorus concentrations at their optimal levels of  $65 \text{ mg l}^{-1} \text{ N}$  and  $8.3 \text{ mg l}^{-1} \text{ P}$ , Fig. 11). *A. junii* had a linear response, i.e. the more potassium was added, the better this bacterium grew, and no optimum was reached within the concentration range of 2 to  $22 \text{ mg l}^{-1} \text{ K}$  (Fig. 11b). *A. baumannii* did not show a response to potassium additions, either.

Table 12. Concentrations of nitrogen (N), phosphorus (P), potassium (K) and magnesium (Mg) giving maximum growth for *Acinetobacter lwoffii*, isolate 9003, *Acinetobacter baumannii*, isolate 910045, and *Acinetobacter junii*, isolate 910093.

Isolate	Concentration (mg l <sup>-1</sup> )					Ratio	
	C	N	P	K	Mg	C:N:P	N:P
<i>A. lwoffii</i> , 9003	(570) <sup>a)</sup>	65	8.3	6.0	6.0	100:11:1.5	7.8
<i>A. baumannii</i> , 910045	570	97	8.2	NE <sup>b)</sup>	NE	100:17:1.4	11.8
<i>A. junii</i> , 910093	(570)	76	8.4	>22	11.7	100:13:1.5	9.1
Average	(570)	79	8.3			100:14:1.5	9.6

<sup>a)</sup>Optimal carbon concentration was determined for *A. baumannii*. This concentration was used also for the other isolates.

<sup>b)</sup>NE = no effect

### 3.5 Phosphate uptake kinetics of four *Acinetobacter* isolates and three other isolates

Control experiments to check whether there was any chemical precipitation or physical adsorption of orthophosphate on to the glassware showed that no phosphate was precipitated or adsorbed during 24 hours up to a pH value of 8.7 in the medium with the trace mineral solution containing EDTA. In the medium without the trace mineral solution, and thus without EDTA, 14 % and 16 % of the orthophosphate precipitated within 4 and 24 hours of incubation, respectively.

#### Phosphate uptake in short term batch cultures (uptake model 1)

A microscopic examination of the Neisser stained cells showed that the bacteria used in the phosphate uptake experiments were almost free from polyphosphate granules.

Judging by the counts of the colony forming units (CFU) no growth took place at least during the first 30 minutes of the experiments, and thus the phosphate taken up was in excess to the normal growth requirements of the cell.

Excess phosphate uptake of the *Acinetobacter* isolates complied with the Michaelis-Menten enzyme kinetic model (Michaelis and Menten 1913). Pooled data of the uptake measurements with *A. lwoffii*, isolate 9003, *A. baumannii*, isolate 910045 and *A. junii*, isolate 910093 produced a maximum initial uptake rate ( $V_{\max}$ ) of 29 mg P g<sup>-1</sup> DM h<sup>-1</sup> and a half-saturation constant ( $K_M$ ) of 17 mg l<sup>-1</sup> P (Fig. 12). The uptake rate (uptake

model 1) can be described by equation (9):

$$v_o = \frac{29 \cdot P_o}{17 + P_o} \quad (9)$$

The  $V_{\max}$  value calculated on the basis of model 1 is not very reliable, since there were only a few measurements corresponding to the high phosphate concentrations.

At initial phosphate concentrations below 10 mg l<sup>-1</sup> P, a linear model ( $v_o = 0.95P_o + 1.3$ ,  $r = 0.848$ ) would result in almost the same rates as model 1. For instance, with initial phosphate concentrations of 2 and 5 mg l<sup>-1</sup> P, which may be found in the influents to the treatment plants of

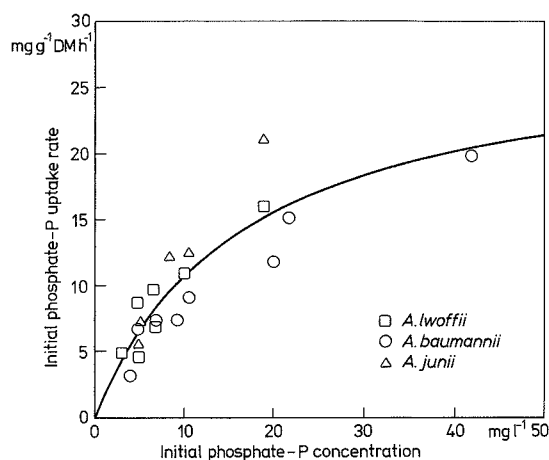


Fig. 12. Initial excess phosphate uptake rates of *Acinetobacter lwoffii*, isolate 9003, *Acinetobacter baumannii*, isolate 910045, and *Acinetobacter junii*, isolate 910093, in short-term batch cultures as a function of the initial phosphate-P concentration in the growth medium.

forest industry, model 1 gives excess phosphate uptake rates of 3.1 and 6.6 mg P g<sup>-1</sup> DM h<sup>-1</sup>, respectively. The linear model would give 3.2 and 6.1 mg P g<sup>-1</sup> DM h<sup>-1</sup>, respectively.

For *A. junii*, 910093, the linear model is  $v_o = 1.3P_o + 0.17$  ( $r = 0.981$ ), for *A. lwoffii*, 9003,  $v_o = 1.1P_o + 1.1$  ( $r = 0.896$ ) and for *A. baumannii*, 910045,  $v_o = 0.83P_o + 0.72$  ( $r = 0.934$ ).

Phosphate uptake rates of the three bacteria which were not acinetobacters although isolated on the acetate mineral medium, were studied by way of comparison. Most of the other non-acinetobacters isolated on the acetate mineral medium did not grow well in the corresponding liquid medium. Sufficient amounts of phosphorus-deficient biomass were acquired only for three isolates. The rates of excess phosphate uptake of these bacteria were low compared with the corresponding rates of acinetobacters, or they did not take up excess phosphate at all.

For *Agrobacter radiobacter*, isolate 920012, the phosphate uptake rate was 2.3 mg P g<sup>-1</sup> DM h<sup>-1</sup> (initial phosphate concentration 10 mg l<sup>-1</sup> P). This isolate did not grow during the 30 min. incubation time, and the uptake could be interpreted to be excess uptake.

The unidentified isolate, 920137, showed no signs of excess phosphate uptake in liquid acetate mineral medium. During the first two hours of incubation there was no phosphate uptake. The uptake rate calculated based on the data of 11 hours of incubation was 0.07 mg P g<sup>-1</sup> DM h<sup>-1</sup> (initial phosphate concentration 8 mg l<sup>-1</sup> P). This uptake was apparently due to growth, since optical densities rose from 0.328 to 0.452 (at 600 nm). After 11 hours the growth rate increased further, and between 11 and 25 hours the phosphate uptake rate was 0.3 mg P g<sup>-1</sup> DM h<sup>-1</sup>.

*Pseudomonas* sp., isolate 920146, had a constant phosphate uptake rate of 0.7 mg P g<sup>-1</sup> DM h<sup>-1</sup> (initial phosphate concentration 8 mg l<sup>-1</sup> P) during 11 hours of incubation. Apparently this uptake was mainly due to growth, since optical densities increased from 0.35 to 1.25 (at 600 nm).

According to uptake model 1 the phosphate uptake rates for acinetobacters at initial phosphate concentrations of 8–10 mg l<sup>-1</sup> P would be 9–11 mg l<sup>-1</sup> P, i.e. almost five-fold in comparison with *Agrobacter radiobacter*, isolate 920012.

Some cells – at the most one third of them – of all three isolates contained violet granules that

were similar to polyphosphate granules, but only lighter. The polyphosphate granules in acinetobacters were usually dark violet, and they were found in the majority or all of the cells.

#### Phosphate uptake during growth in a batch culture (uptake model 2)

In these experiments phosphate uptake was due to the growth of bacteria and to polyphosphate formation. The microscopic examination of the Neisser stained cells showed that polyphosphate formation took place during the growth phase (Fig. 13).

The growth and phosphate uptake kinetics of the batch cultures of the four *Acinetobacter* isolates studied can be presented with uptake models 2a–d (equations 10–17; for the symbols and dimensions of the parameters, see chapter 2.8):

For *A. lwoffii* (9003), model 2a

$$\frac{dP}{dt} = -\frac{0.55}{4.1} \cdot \frac{P}{(6.0 + P)} \cdot B \quad (10)$$

$$\frac{dB}{dt} = 0.55 \cdot \frac{P}{(6.0 + P)} \cdot B \quad (11)$$

For *A. baumannii* (910045), model 2b

$$\frac{dP}{dt} = -\frac{0.35}{3.6} \cdot \frac{P}{(4.0 + P)} \cdot B \quad (12)$$

$$\frac{dB}{dt} = 0.35 \cdot \frac{P}{(4.0 + P)} \cdot B_{t-1} \quad (13)$$

For *A. junii* (910093), model 2c

$$\frac{dP}{dt} = -\frac{0.20}{4.5} \cdot \frac{P}{(2.5 + P)} \cdot B \quad (14)$$

$$\frac{dB}{dt} = 0.20 \cdot \frac{P}{(2.5 + P)} \cdot B_{t-1} \quad (15)$$

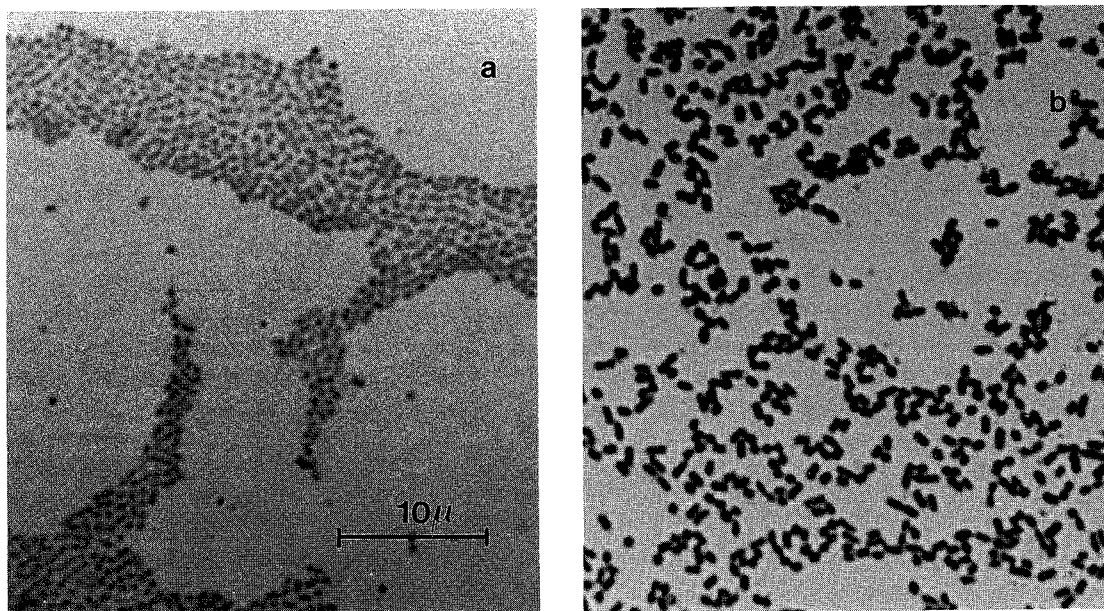


Fig. 13. Neisser stained cells of *Acinetobacter junii*, isolate 920121, at the beginning of the experiment (a) and during growth, 18 hours after the inoculation (b).

For *A. junii* (920121), model 2d

$$\frac{dP}{dt} = -\frac{0.15}{6.2} \cdot \frac{P}{(0.40 + P)} \cdot B \quad (16)$$

$$\frac{dB}{dt} = 0.15 \cdot \frac{P}{(0.40 + P)} \cdot B_{t-1} \quad (17)$$

A time delay of one hour between phosphate uptake and growth was included in models 2b – d.

The curves in Fig. 14a – d present an increase in the cell concentration and a decrease in the phosphate concentration of the growth medium as calculated by models 2a – d. The models fit well to the data except for *A. junii*, 920121 at the end of the experiment. This isolate continued to grow at the expense of its internal polyphosphate storage after phosphate had been exhausted in the growth medium. The Michaelis-Menten model does not take the internal storage into account, and thus it predicts erroneously a slight increase in the phosphate concentration. This increase was, however, too small to be detected in the scale of Fig. 14d.

According to uptake models 2a – d, the uptake rate for an initial phosphate concentration of 2 mg l<sup>-1</sup> P is on an average 2.6 mg P g<sup>-1</sup> DM h<sup>-1</sup> (Table 13). Variation between the uptake rates of the *Acinetobacter* isolates at low phosphate concentrations was slight. *A. baumannii* had the lowest uptake rates at low concentrations.

*A. lwoffii* had the highest (12 mg P g<sup>-1</sup> DM h<sup>-1</sup>), and *A. junii*, isolate 920121 the lowest (3.7 mg P g<sup>-1</sup> DM h<sup>-1</sup>) maximum uptake rates. It can be calculated by models 2a – d that *A. junii*, 920121, would approach its maximum uptake rate at initial phosphate concentrations around 10 mg l<sup>-1</sup> P, while the other isolates would need about 100 mg l<sup>-1</sup> P to reach this uptake rate.

The differences between the isolates were also reflected in the half-saturation constants ( $K_s$ ).  $K_s$  was 6.0 mg l<sup>-1</sup> P for *A. lwoffii* and 0.40 mg l<sup>-1</sup> P for *A. junii*, isolate 920121.

The maximum specific growth rates ( $\mu_{max}$ ) varied almost four-fold, with *A. lwoffii* having the highest (0.55 h<sup>-1</sup>) and *A. junii*, isolate 920121 the lowest (0.15 h<sup>-1</sup>) rates.

The ratios  $\mu_{max}/K_s$  and  $V_{max}/K_s$ , which can be used as indicators of the ability of a bacterium to compete for low substrate concentrations (Healey



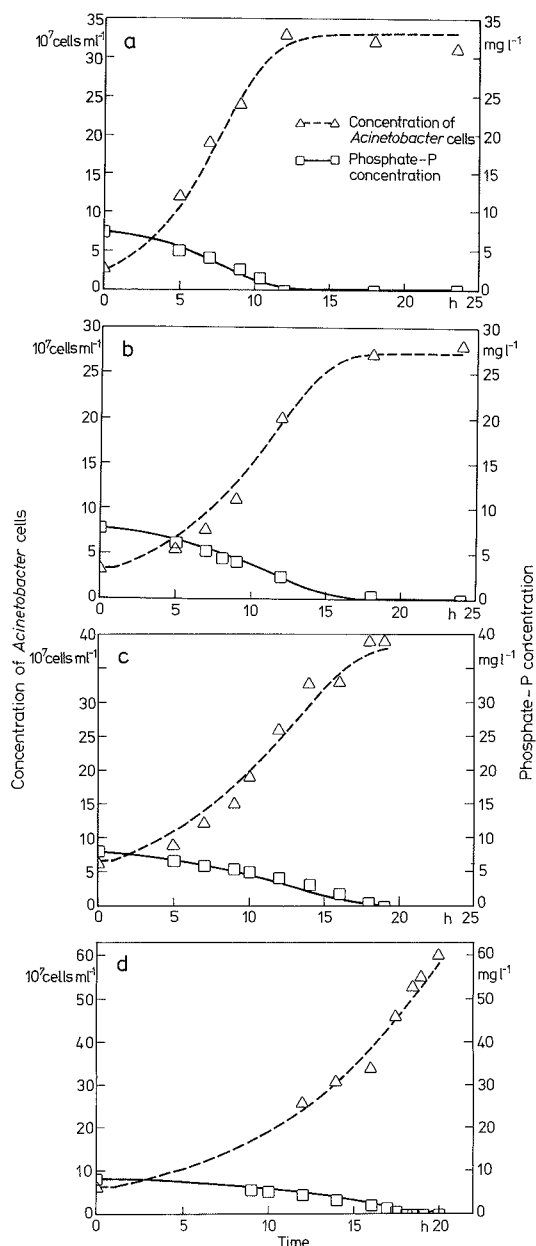


Fig. 14a – d. Phosphate uptake during growth. The cell number ( $10^7 \text{ cells ml}^{-1}$ ) and phosphate uptake (shown by the decreasing phosphate concentrations in the growth medium,  $\text{mg l}^{-1} \text{ P}$ ) of (a) *Acinetobacter lwoffii*, isolate 9003, (b) *Acinetobacter baumannii*, isolate 910045, (c) *Acinetobacter junii*, isolates 910093 and (d) 920121. The curves present the growth and uptake as calculated by models 2a – d. Note the difference in scales.

1980, Lobry et al. 1992), are presented in Table 14.

The yield coefficients of the isolates (W, Table 13), which give the number of cells needed to take up a phosphate unit, varied less than other parameters between the isolates, ranging from  $3.6$  to  $6.2 \cdot 10^7 \text{ cells } \mu\text{g}^{-1} \text{ P}$  or, expressed per dry cell mass, from  $34.7$  to  $64.1 \text{ mg DM mg}^{-1} \text{ P}$ . For *A. junii* isolates 910093 and 920121, more cells and less dry cell mass was needed for the uptake of a given amount of phosphate. The cells of both *A. junii* isolates were smaller (small coccoid rods) than the cells of *A. lwoffii* (long, thin rods) and *A. baumannii* (big rods), which is reflected by the factor (b) converting the number of cells into dry mass.

### Phosphorus content of the biomass

The total phosphorus concentrations analyzed in the biomass grown under phosphate limitation varied from  $0.5$  to  $1.1 \%$  of dry cell mass, being most often close to  $1 \%$ .

The total phosphorus concentrations analyzed at the end of the phosphate uptake experiments with the growing biomass varied between  $1.3$  and  $1.7 \%$  (Table 15). The corresponding estimates based on phosphate uptake (initial phosphate concentrations of  $8 \text{ mg l}^{-1} \text{ P}$ ) were higher, varying from  $2.7$  to  $3.6 \%$ .

Variations in the phosphorus content of the biomass during one uptake experiment were small, as well as variations between the *Acinetobacter* isolates.

Based on the biomass concentration and the decrease of the phosphate concentration in the medium it can be estimated that after short-term incubation the phosphorus content of the biomass varied between  $1.1$  and  $4.2 \%$  (the phosphorus content of  $0.5 - 1 \%$  before the experiment), depending on the ratio of the biomass and phosphate concentrations in the medium.

### 3.6 Phosphate release kinetics of five *Acinetobacter* isolates

Phosphate release was linear in relation to time. No indication of cell lysis could be detected under the microscope during the anaerobic period.

Table 13. Phosphate uptake rates ( $v$ ) at initial phosphate concentrations ( $P_0$ ) of 2 and 5 mg l<sup>-1</sup> P, maximum uptake rate of phosphate ( $V_{\max}$ ), maximum specific growth rate ( $\mu_{\max}$ ), half-saturation constant ( $K_s$ ) and yield coefficient ( $W$ ) of four *Acinetobacter* isolates. Coefficient  $b$  relates cell density to dry mass (DM).

Isolate	$P_0$ (mg l <sup>-1</sup> )	$v$ (mg P g <sup>-1</sup> DM h <sup>-1</sup> )	$V_{\max}$ (mg P g <sup>-1</sup> DM h <sup>-1</sup> )	$\mu_{\max}$ (h <sup>-1</sup> )	$K_s$ (mg l <sup>-1</sup> P)	$b$ ( $\mu$ g DM (10 <sup>7</sup> cells) <sup>-1</sup> )	$W$	
							(10 <sup>7</sup> cells $\mu$ g <sup>-1</sup> P)	(mg DM mg <sup>-1</sup> P)
<i>A. lwoffii</i> , 9003	2	3.0	12	0.55	6.0	11.4	4.1	46.7
	5	5.4						
<i>A. baumannii</i> , 910045	2	1.8	5.5	0.35	4.0	17.8	3.6	64.1
	5	3.0						
<i>A. junii</i> , 910093	2	2.6	5.8	0.20	2.5	7.7	4.5	34.7
	5	3.8						
<i>A. junii</i> , 920121	2	3.1	3.7	0.15	0.4	6.5	6.2	40.3
	5	3.4						
Average	2	2.6	6.8	0.31	3.2	10.9	4.6	50.1
	5	3.9						

Table 14. The ratios of the maximum specific growth rate ( $\mu_{\max}$ ) and the maximum phosphate uptake rate ( $V_{\max}$ ) to the half-saturation constant ( $K_s$ ) for four *Acinetobacter* isolates.

Isolate	$\mu_{\max}/K_s$ (l mg <sup>-1</sup> h <sup>-1</sup> P)	$V_{\max}/K_s$ (l g <sup>-1</sup> DM h <sup>-1</sup> )
<i>A. lwoffii</i> , 9003	0.092	2.0
<i>A. baumannii</i> , 910045	0.088	1.4
<i>A. junii</i> , 910093	0.080	2.3
<i>A. junii</i> , 920121	0.375	9.3

Moreover, significant lysis would have been detected as increasing release rates at the end of the experiment.

After short-term aerobic incubation the amount of phosphate released (as P) during the anaerobic phase varied between 0 % and 70 %, depending on the *Acinetobacter* isolate and on pH (Table 16). *A. baumannii*, isolate 910045, did not release phosphate at pH 7.0, whereas at pH 6.0, 16 % of the phosphorus bound under aerobic conditions was released (Fig. 15a and b). *A. junii*, isolate 920121, released more than half of the phosphate bound irrespective of the pH value. At

pH 7.0, 55 % during a 21 h and 63 % during a 24 h incubation was released, assuming a constant release rate. At pH 6.0, 61 – 70 % was released during a 24 h incubation (Fig. 15c and d). *A. junii*, isolate 910093, and *A. lwoffii*, isolate 920122, released more phosphate (27 % and 26 % respectively) than *A. baumannii*, isolate 910045, but clearly less than *A. junii*, isolate 920121.

The release rates at pH 6.0 varied about 10-fold from 0.033 mg P g<sup>-1</sup> DM h<sup>-1</sup> for *A. baumannii* to 0.26 mg P g<sup>-1</sup> DM h<sup>-1</sup> for *A. junii*, isolate 920121.

When anaerobic conditions were introduced after a growth period, the amounts released were smaller than in the corresponding short-term batch cultures (experiments were made only at pH 6.0). For *A. junii*, isolates 910093 and 920121, the percentages of phosphate release after growth were 9 % and 27 %, respectively, which are about one third of the corresponding percentages obtained in the short-term incubation tests. The percentage was 10 % for *A. baumannii* (almost two thirds compared with the other type of experiment), and 8 % for *A. lwoffii*, isolate 9003.

The release rates after the growth period were, however, about the same as ( $0.21 \text{ mg P g}^{-1} \text{ DM h}^{-1}$  for *A. junii*, 920121) or even higher than ( $0.067 \text{ mg P g}^{-1} \text{ DM h}^{-1}$  for *A. baumannii*, 910045) in the corresponding short-term batch cultures. The rate for *A. lwoffii*, 9003 was  $0.059 \text{ mg P g}^{-1} \text{ DM h}^{-1}$ , which was close to the rate obtained in the short-term culture for *A. lwoffii*, isolate 920122.

Table 15. Estimated and measured total phosphorus contents of *Acinetobacter* biomass in the growing batch cultures at different sampling times. The initial concentration of phosphate in the acetate mineral medium was  $8 \text{ mg l}^{-1} \text{ P}$ .

Isolate and sampling time (h)	Phosphorus content of the biomass <sup>a)</sup> (% of dry cell mass)
<i>A. lwoffii</i> , 9003	
0	(1.1) <sup>b)</sup>
5	2.9
9	2.7
12	3.1
18	3.1
23.5	3.2 (1.7)
<i>A. baumannii</i> , 910045	
0	(1.1)
5	2.8
7	3.0
9	3.0
12	2.6
18	2.7
24	2.7 (1.3)
<i>A. junii</i> , 910093	
0	(0.9)
5	3.1
7	3.3
9	3.3
10	3.1
12	2.9
16	3.4
18	3.5
19	3.6 (1.3)
<i>A. junii</i> , 920121	
0	(1.1)
12	3.2
14	3.4
16	3.8
17.5	3.6
18.5	3.4 (1.6)
19	3.3
20	3.1 (1.5)

a) The amount of phosphate taken up by a biomass unit is added to the amount analyzed at time 0 h.

b) The figures in parenthesis are results of the chemical analyses (SFS 3026) of the total phosphorus content of the biomass.

It was found that phosphate uptake continued under anaerobic conditions as well (resazurin indicated that the  $E_h$  was  $-110 \text{ mV}$  or less) if anaerobiosis was introduced when there was still some phosphate left in the medium ( $1 - 2 \text{ mg l}^{-1} \text{ P}$ ).

In the growing, aerobic culture of *A. lwoffii*, isolate 9003, some phosphate release was observed already under aerobic conditions after the phosphate concentration in the medium had dropped close to zero (phosphate concentration in the medium was  $0.04 \text{ mg l}^{-1} \text{ P}$  after 12 h and  $0.14 \text{ mg l}^{-1} \text{ P}$  after 23.5 h aerobic incubation).

The amounts of phosphorus released, calculated on the basis of the total phosphorus contents analyzed in the biomass, were in some cases close to those calculated on the basis of phosphate uptake and release from and into the medium, but there were also deviations.

Microscopic examination of the Neisser stained cells showed that many cells still contained polyphosphate granules at the end of both types of release experiments. There were more ungranulated cells in the cultures made anaerobic after the growth period than in those after the short-term incubation.

### 3.7 Denitrification potential of *Acinetobacter* isolates

None of the 57 *Acinetobacter* isolates converted nitrate to  $\text{N}_2\text{O}$ . The percentages of nitrate converted to gas were at the same level as in the uninoculated control tubes (0.0002 %).

## 4 DISCUSSION

### 4.1 *Acinetobacter* in wastewater and activated sludge

There are many bacteriological studies concerning activated sludge treating municipal wastewaters (chapter 1.4.1), but only a few have been undertaken on bacterial populations in wastewater and activated sludge of forest industry treatment plants. The studies performed on forest

industry process— and wastewaters have usually been concerned with bacteria belonging to the family *Enterobacteriaceae* (Knowles et al. 1974, Neilson and Sparell 1976, Niemi et al. 1987).

Polyphosphate accumulating bacteria (poly-P bacteria) in wastewater treatment plants of pulp and paper mills have not been investigated before

the present study. Isoaho et al. (1981) studied the activated sludge of a pilot plant designed for treating wastewaters from the bleaching and evaporation phases of sulphite mills, but microbes were not studied in detail. Väättä and Niemelä (1982) studied bacterial populations in the process waters of a paper mill, observing also the

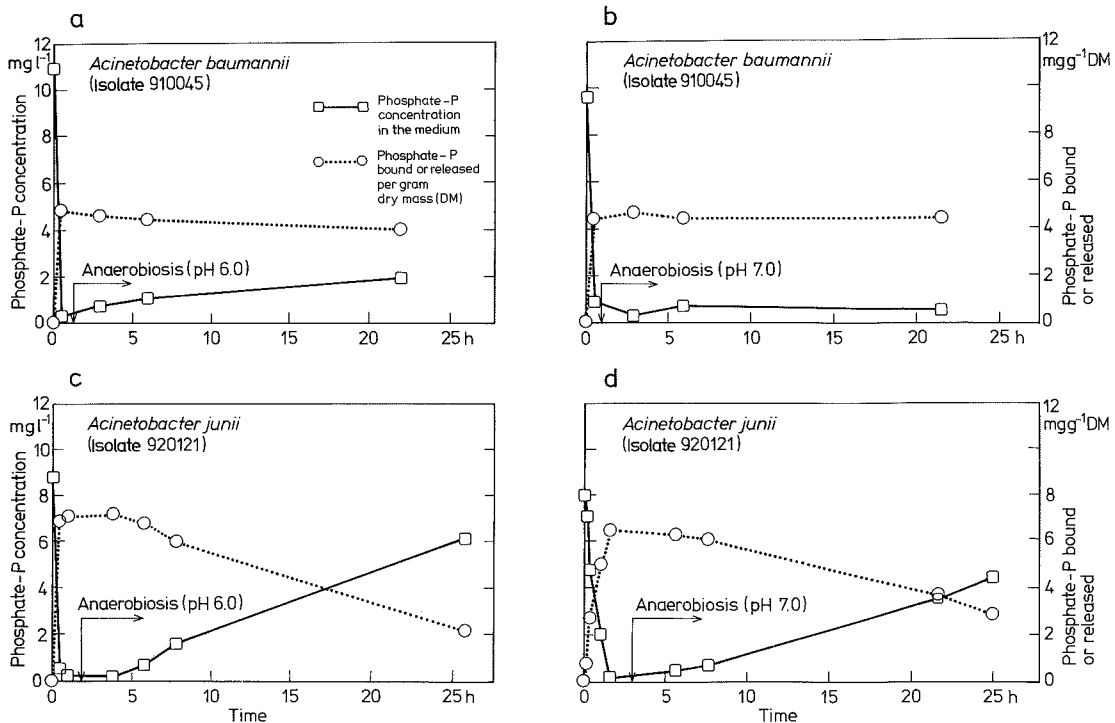


Fig. 15a – d. Uptake and release of phosphate (shown by changes in phosphate concentrations of the growth medium,  $\text{mg l}^{-1} \text{P}$ , and by phosphate bound or released per gram dry cell mass,  $\text{mg P g}^{-1} \text{DM}$ ) for *Acinetobacter baumannii*, isolate 910045 (a and b) and *Acinetobacter junii*, isolate 920121 (c and d). The pH in the anaerobic phase was 6.0 (a and c) or 7.0 (b and d).

Table 16. Phosphorus release rate per dry cell mass and percentage of phosphorus released in relation to phosphorus bound by *Acinetobacter* isolates.  $P_0$  is the phosphate concentration at the beginning of the aerobic phase of the short-term batch experiment. The pH was 6.0 if not otherwise stated.

Isolate	$P_0$ ( $\text{mg l}^{-1}$ )	Release rate ( $\text{mg P g}^{-1} \text{DM h}^{-1}$ )	P released per P bound (%)
<i>A. baumannii</i> (910045)	11	0.033	16 <sup>a)</sup>
	10	0	0 <sup>a)</sup> (pH 7.0)
<i>A. junii</i> (910093)	8	0.052	27 <sup>b)</sup>
<i>A. junii</i> (920121)	9	0.22	70 <sup>b)</sup>
	8	0.19	55 <sup>a)</sup> (pH 7.0)
	17	0.26	61 <sup>b)</sup>
<i>A. lwoffii</i> (920122)	8	0.072	26 <sup>b)</sup>

a) During 21 – 22 hours.

b) During 24 hours

presence of acinetobacters.

One of the objectives of this study was to investigate the bacterial populations in forest industry wastewaters and activated sludge treating these wastewaters, paying special attention to acinetobacters. Acinetobacters are usually considered to be the most important of the poly-P bacteria, although their role has also been questioned (chapter 1.4.1). The significance of acinetobacters for phosphorus removal in the present aerobic activated sludge treatment processes of forest industry wastewaters will be evaluated in chapter 4.3.

The number of colonies identified per one sample (10 – 20) was too small for statistical analysis. The main objective was to screen samples of different treatment plants to find out whether significant numbers of acinetobacters were present. This kind of screening revealed differences between samples, for instance there was a marked difference between the two samplings of pulp mill 2. Because the colonies to be identified were picked from plates spread with the most diluted samples, they were likely to represent the majority of bacteria growing on the acetate mineral medium.

#### Selectivity of the acetate mineral medium for acinetobacters and applicability of the API 20 NE identification kit

Acetate mineral medium is considered to be selective for acinetobacters, and therefore their presumptive numbers are estimated on the basis of colony forming units on this medium (Hiraishi et al. 1989a). However, the present study shows clearly that the number of acinetobacters cannot even roughly be estimated on the basis of colonies growing on the acetate mineral medium.

Only 15 % of the 372 colonies isolated on the acetate mineral medium were identified as acinetobacters with the API 20 NE identification kit. Most of the bacteria (59 %) could not be identified with this system. The acetate mineral medium sustained the growth of many isolates identified for instance as *Pseudomonas*, *Pasteurella* and *Chryseomonas* (Tables 10 and 11). The identifiable isolates belonged to 21 different genera or species. Three *Acinetobacter* species (*A. lwoffii*, *A. baumannii* and *A. junii*) were identified.

Acinetobacters grow rapidly on acetate mineral

medium. The increase in the number of small pin-point colonies after 2 – 3 days of incubation indicates that under prolonged incubation many other bacteria can grow on this medium. This is reflected in the increase in the ratio of colonies on acetate plates to colonies on TGY plates (A/TGY % in Table 10) with increasing incubation times.

The API 20 NE identification system provided acceptable identification only for a minority of cultivable bacteria in activated sludge. Apparently, this holds for environmental samples in general (Breschel and Singleton 1992). On the other hand, the API 20 NE identification kit seemed to be suitable for identifying acinetobacters within the present taxonomic framework. In repeated identifications the same result was obtained, provided that the identification level (chapter 2.4) was at least acceptable. The isolates identified with "low discrimination" (classified as non-identifiable bacteria in this study) did not always produce the same identification profile in replicate tests, due to some physiological trait providing variable reactions from time to time.

However, it must be borne in mind that the whole taxonomy of acinetobacters is obscure (chapter 1.4.1). Therefore it may not be worthwhile to pay too much attention to the identification results at the species level. It seems more important to confirm that the isolate is *Acinetobacter*, a potential polyphosphate accumulator, and then test its ability to accumulate excess phosphate. A promising method for monitoring purposes is determination of the enzyme activity of adenylate kinase, which catalyzes polyphosphate degradation in bacteria (van Groenestijn et al. 1989a).

#### Acinetobacters and other potential polyphosphate-accumulating bacteria

Whenever acinetobacters could be isolated from the most diluted samples, their numbers were estimated to be between  $0.1 \cdot 10^6$  and  $9 \cdot 10^6$  CFU ml<sup>-1</sup> (colony counts on the acetate mineral medium, corrected with the observed frequencies of acinetobacters). These figures are of the same order of magnitude as those found by Cloete et al. (1985) in municipal treatment plants with anaerobic and aerobic zones.

In most sludge samples acinetobacters constituted less than 2 % of the aerobic heterotrophic

bacterial population cultivable on the TGY (1:5) medium. This percentage is at the lower end of the range reported by Brodisch and Joyner (1983), Cloete and Steyn (1988a) and Hiraishi et al. (1989a) in laboratory- and pilot-scale activated sludge units with enhanced biological phosphorus removal. Brodisch and Joyner (1983) found that *Acinetobacter* sp. constituted 1 – 10 % of the total cultivable bacterial population (casitone-glycerol-yeast extract medium), with *Aeromonas*, *Pseudomonas* and Gram-positive bacteria being dominant. Bacterial populations were not very different in the various stages of the process with different redox potentials. In the study of Hiraishi et al. (1989a), *Acinetobacter* accounted for 1 % at the most (colonies growing on acetate mineral medium and identified on the basis of respiratory quinone profiles) of the total cultivable population (polypeptone-yeast extract medium). The majority of the population consisted of *Comamonas*, *Pseudomonas*, *Paracoccus* and the *Flavobacterium-Cytophaga* group. On the basis of direct microscopic counts Cloete and Steyn (1988a) estimated that *Acinetobacter* constituted less than 10 % of the total bacterial population in the sludge.

The total number of bacteria cultivable on the TGY (1:5) medium was of the same order of magnitude as generally reported for sludge samples (e.g. Hiraishi et al. 1989a). Nevertheless the actual total number of bacteria is likely to be at least 10 times the number growing on any artificial medium (e.g. Cloete and Steyn 1987). On the other hand, artificial media select for the gamma-subclass of proteobacteria, such as *Acinetobacter* which can be quantitatively detected on culture media (Toerien et al. 1990, Wagner et al. 1993). In view of this information, it is likely that acinetobacters constituted only minor fractions of a percent of the total bacterial population of the activated sludge studied.

Phosphorus removal by the sludge is a function of the number and phosphorus uptake rate of bacteria. Brodisch and Joyner (1983) and Hiraishi et al. (1989a) did not study the phosphorus uptake rates of their isolates, and therefore it remains uncertain which bacteria were mainly responsible for the observed phosphorus removal. Cloete et al. (1985) concluded on the basis of the polyphosphate content of *Acinetobacter* cells that these bacteria ( $1.4 \cdot 10^6 - 6.5 \cdot 10^6$  CFU ml<sup>-1</sup>) could not have accounted for the phosphorus

removal. However, even a relatively small number of acinetobacters may take up most of the phosphorus if the uptake rate is high.

Among the isolates on the acetate mineral medium there were also other potential polyphosphate accumulators than acinetobacters; for instance *Pseudomonas*, *Flavobacterium* and *Moraxella* (low discrimination from *Pseudomonas diminuta*; classified as non-identifiable in Tables 10 and 11) occurred frequently. All these bacteria have been reported to accumulate polyphosphates (*Pseudomonas* and *Moraxella*, Lötter and Murphy 1985, Suresh et al. 1985, Okada et al. 1992; *Flavobacterium-Cytophaga* group, Hiraishi and Morishima 1990).

There were many isolates with a low discrimination between *Acinetobacter* and some other bacterium (e.g. *Pseudomonas*). Judging by their resemblance to acinetobacters, these bacteria are also potential polyphosphate accumulators.

The polyphosphate accumulating ability of the *Pseudomonas* species would be worth studying. In the present study the phosphate uptake of one *Pseudomonas* isolate (920146) was investigated (chapters 3.5 and 4.3).

Almost all of the isolates on the acetate mineral medium were Gram-negative bacteria. The occurrence and significance of Gram-positive bacteria in the total bacterial population of sludge treating forest industry wastewaters should be studied in detail. Hiraishi and Morishima (1990) found that unidentified Gram-positive cocci were the most efficient polyphosphate accumulators in a laboratory-scale batch reactor, but they constituted only a minority of the bacterial population.

Toxicity of pulp and paper wastewaters to acinetobacters was not studied specifically, but apparently these wastewaters were not toxic to acinetobacters, since these bacteria could be isolated in the influents. Acinetobacters can also be expected to be tolerant to toxic substances, since some of them are known to be able to degrade various recalcitrant compounds (Asperger and Kleber 1991, Fewson 1991, Gutnick et al. 1991).

#### Effects of nitrogen level and influent acinetobacters on the occurrence of acinetobacters in sludge

According to Lammi and Pakarinen (1993), removal of phosphorus and organic matter was

improved during a period when nitrogen was added to the wastewater treatment process of pulp mill 2. With added nitrogen the phosphorus reduction was 74 % and without it 47 %. In the present study half of the total cultivable bacteria were acinetobacters (Table 10) in the influent sample of this treatment plant during a nitrogen addition period (not the same period as studied by Lammi and Pakarinen, 1993). In the sample taken when nitrogen was not added, no acinetobacters could be isolated. The absence of acinetobacters among the dominant cultivable bacteria may be due either to the low nitrogen level or the low pH value (4.6) of the influent; during nitrogen addition pH was 5.7. Nitrogen level and pH were the only factors known to be different in the two samplings of this treatment plant. There were no major differences in the quality and quantity of pulp production, nor were there any disturbances in the treatment process during the study period.

In the treatment plant of pulp mill 2, nitrogen was added before the equalization basin, where the wastewater was retained for a few hours. The samples were taken after this stage, and this leaves room for speculation whether the equalization basin served as an enrichment reactor for acinetobacters. For instance in the treatment plant of paper mill 1 nutrients were added immediately before the wastewater entered the aeration basin, leaving no time for enrichment.

Although dominant in the influent to the treatment plant of pulp mill 2, acinetobacters were not among the most common bacteria in the sludge. This indicates that the *Acinetobacter* population entering the treatment plant with the influent was not enriched in the aeration basin. In the sludge, non-identifiable bacteria and *Pseudomonas* were dominant, i.e. the situation did not differ from the one in which acinetobacters could not be isolated from the influent.

During the first sampling of the treatment plant of paper mill 2, two weeks before the nitrogen addition was halved, acinetobacter numbers were fairly high both in the influent and the return sludge (Table 10). In this case acinetobacters seemed to be present throughout the treatment plant. Detection of acinetobacters became increasingly difficult with decreasing nitrogen additions, and no acinetobacters were isolated among the dominant bacteria of the sludge after four months without nitrogen

additions.

Obviously the conditions in the treatment plant determine whether the influent bacteria, or any other inoculum, survive and multiply in the sludge. If the treatment plant is operated so that acinetobacters achieve competitive advantage over other aerobic bacteria, influent bacteria or a commercial inoculum could facilitate the creation of a suitable bacterial flora in the sludge, for instance after an operational failure in the treatment plant. Meloni (1991) has suggested that acinetobacters in the white water of a paper machine might have facilitated a fast onset of phosphorus removal.

The effects of nitrogen levels on the occurrence of acinetobacters in the treatment plants of pulp and paper mills would be an important subject for closer studies. The effects of nitrogen on the growth of *Acinetobacter* pure cultures is discussed in chapter 4.2.

### Enrichment of acinetobacters

In most samples it was easy to enrich acinetobacters within a week in well-aerated liquid acetate mineral medium. Under these conditions acinetobacters became dominant in some samples. Approximately one third of the isolates (94) in the enrichment cultures were identified as acinetobacters. This is three times the percentage found in the non-enriched samples (11 %). *Pseudomonas* (21 % of the isolates in the enrichment cultures) and non-identifiable bacteria (50 %) were numerous in the enrichment cultures as well.

Acinetobacters were also enriched in the influent and activated sludge of pulp mill 1, in which they could not be detected by cultivating samples directly without prior enrichment (Puustinen and Jørgensen, unpublished results). The results show that acinetobacters are also present in the activated sludge treating pulping wastewaters, although their numbers may be too low to be detected in the most diluted sludge samples. This would mean that enhanced biological phosphorus removal may be successful in pulp mill wastewaters if the process is designed so as to favour acinetobacters.

According to Grimont and Bouvet (1991), *A. baumannii* has been found only in humans. However, in the present study this species was

found in the enrichment cultures of activated sludge in particular. Whether this is due to the inability of the API 20 NE system to identify the species correctly or to incomplete knowledge of the ecology of *Acinetobacter* remains to be demonstrated. It is possible, moreover, that *A. baumannii* originates from the sanitary system of the mill and occurs in small numbers in the sludge, where it is easily enriched.

## 4.2 Optimal growth conditions for three *Acinetobacter* isolates

### Carbon

Volatile fatty acids (formic acid, acetic acid, propionic acid and butyric acid) are important in the carbon and polyphosphate metabolism of acinetobacters (chapter 1.4.2). In wastewater treatment these acids are produced by fermentative bacteria from organic compounds in wastewater and sludge, or they may be present in the influent wastewater itself. The fermentative phase can be a rate-limiting factor for enhanced phosphorus removal (van Starkenburg et al. 1993).

According to Pitman (1991) enhanced biological phosphorus removal requires "readily biodegradable COD" (COD is chemical oxygen demand) or volatile fatty acids in concentrations higher than 100 mg l<sup>-1</sup>. If the concentration is lower than 50 mg l<sup>-1</sup>, special attention must be given to process design. Pitman (1991) does not specify exactly what is meant by the concentrations "mg l<sup>-1</sup>", but if they are COD-values of acetic acid, these concentrations correspond to 38 mg l<sup>-1</sup> C and 19 mg l<sup>-1</sup> C, respectively. Winter (1989) has found that an acetate concentration of 58 mg l<sup>-1</sup> C results in efficient phosphorus removal.

The high molecule-weight lignin compounds, which form a substantial part of the organic carbon compounds in pulping wastewaters, are not readily biodegradable and can hardly be fermented to volatile fatty acids within the time that the sludge remains in a primary settler of a conventional activated sludge treatment plant. For instance in the treatment plant of pulp mill 2 (sulphate pulp), half of the dissolved organic carbon was not degraded in the aeration basin

(Table 8 in chapter 3.1).

In the influents of pulp mill 1 (sulphate pulp), the total concentration of volatile fatty acids was less than 20 mg l<sup>-1</sup> C (Table 7, chapter 3.1). Judging by the results of this mill, the lack of suitable carbon compounds is likely to be an important factor limiting the growth of acinetobacters in treatment plants of sulphate pulp mills. If enhanced biological phosphorus removal is to be achieved in the wastewater treatment of sulphate pulp mills, it seems necessary to generate suitable carbon compounds by fermenting part of the sludge together with the influent either in primary settling tanks or in separate anaerobic digesters. Direct additions of volatile fatty acids would have the same effect, but this would be expensive.

In the chemo-thermomechanical pulping with alkaline pre-treatment, acetic acid dissolves into the wastewater. Acetic acid is also formed in the alkaline peroxide bleaching of mechanical pulps (Virkola and Honkanen 1985). Furthermore, it has been observed that clarified white water from thermomechanical pulp production is readily degradable into volatile fatty acids (Rintala and Vuoriranta 1988).

Because the manufacturing of mechanical pulps is integrated with a paper or board mill, wastewater from paper mills may contain significant amounts of acetic acid. Indeed, the concentrations of volatile fatty acids in the wastewater from the three paper mills studied were fairly high (60 – 170 mg l<sup>-1</sup> C, Table 7 in chapter 3.1), being clearly above the values of 38 mg l<sup>-1</sup> C and 58 mg l<sup>-1</sup> C given by Pitman (1991) and Winter (1989), respectively, for successful biological phosphorus removal. Results of the treatment plant of paper mill 3 indicate that fermentation took place in the primary sedimentation basin. The concentration of volatile fatty acids in the primary sedimentation basin was 51 mg l<sup>-1</sup> C and in the following equalization basin 96 mg l<sup>-1</sup> C.

Although paper mill wastewaters may contain fairly high concentrations of acetic acid, a fermentative phase could be recommendable for the treatment of this kind of wastewater as well. The fermentative phase has been found to improve phosphorus removal in the treatment of municipal wastewaters containing high concentrations of readily biodegradable organic carbon compounds (Buchan 1982, 1983, Mulder and



Rensink 1987, Winter 1989, Abu-gharah and Randall 1991, Pitman et al. 1992).

It can be calculated that a volatile fatty acid concentration of  $170 \text{ mg l}^{-1} \text{ C}$ , which was the highest concentration in the paper mill influents studied, could sustain an *Acinetobacter* biomass of about  $120 \text{ mg l}^{-1}$  dry cell mass, which corresponds to a cell number of  $2 \cdot 10^8 \text{ cells ml}^{-1}$  (calculation is based on the data of optimization experiment 1, chapter 3.4, and on the relation between the optical density, the dry mass and microscopic counts of the bacteria obtained in this study). In addition to volatile fatty acids, the influents should, of course, contain enough of other essential growth factors as well, and the conditions in the treatment plant should favour the survival of the influent *Acinetobacter* population. In practice, many other bacteria also compete for the volatile fatty acids.

### Nitrogen and phosphorus

In wastewater treatment the optimal C:N:P ratio may be more important than the optimal concentrations of these nutrients. Stanier et al. (1975) and Fenchel and Blackburn (1979) give average C:N:P ratios (on a weight basis) of 100:28:6 and 100:18:6 for bacterial cells, respectively. These ratios are derived from the chemical composition of the cell. Optimal growth of the *Acinetobacter* isolates was obtained with a C:N:P ratio of 100:14:1.5 in the growth medium as an average of all three isolates (Table 12 in chapter 3.4).

By converting acetate-carbon to COD the ratio COD:N:P = 100:5:0.5. When comparing this ratio with the corresponding ratios measured in the treatment plants it has to be borne in mind, however, that the  $\text{COD}_{\text{C}}$  value measured in the wastewater encompasses all the inorganic and organic compounds that are oxidizable with dichromate and therefore overestimates the biologically available carbon pool.

The ratios of C:N:P or any other elements (c.f. results of optimal Mg and K concentrations) obtained by determining the chemical composition of the biomass represent a "static" situation after the growth has ceased, and thus equal the net uptake of the elements. The chemical composition of a microorganism depends on its growth rate and environment

(Abbot et al. 1973). Part of the carbon taken up from the growth medium is respired and thus not included in ratios of this kind. These ratios are therefore not directly comparable with the ratios obtained by determining the combination of elements that gives the best growth. The latter reflect the utilization and turnover of the elements rather than mere net uptake.

Assuming that the amount of carbon respired by the biomass is 50 % and all nitrogen and phosphorus taken up remain in the cell, the carbon to nutrients ratio of the biomass, as presented by Stanier et al. (1975) and Fenchel and Blackburn (1979), would be 100:(9 - 14):3 in the growth medium. As regards the ratio of carbon to nitrogen, this ratio is close to the ratio obtained for acinetobacters. The phosphorus requirement of acinetobacters was, however, lower than presented in the literature. The nutrient requirements of acinetobacters were the same as those obtained for *Aerobacter aerogenes* (100:15:1.5) growing in a chemostat (Dean and Rogers 1967).

Maximum growth of acinetobacters was obtained with an average N:P ratio of 10 in the medium (on a weight basis). This indicates that the growth of acinetobacters could probably be enhanced with a higher N:P ratio than has been traditionally used in wastewater treatment (about 5, ranging from 3 to 8; Naakka et al. 1970, Cornacchio and Hall 1988, Meloni 1991). The effects of the higher N:P ratio on other characteristics of the sludge, for instance on flocculation or formation of filaments, should be carefully investigated before considering increased nitrogen levels.

Although the nitrogen to phosphorus ratio of acinetobacters was higher than generally presented in the literature for bacteria, it was well within the range presented for planktonic algae. For algae the molar N:P ratio where one nutrient limitation changes for the other, defined as the optimal ratio, has been reported to range from 7 to 53 (Cembella et al. 1984). These molar ratios varied from 17 to 27 for the *Acinetobacter* isolates, averaging 21.

As suggested by Puhakka (1990), a practical way of balancing the high carbon to nutrient ratio of forest industry wastewaters could be to recycle some of the nutrient-rich filtrate obtained in the anaerobic digestion of sludge, which is done for stabilizing the sludge for landfill disposal.

The ratios based on the composition of the

growth medium provide a better basis for optimizing the growth conditions of bacteria than the ratios based on the chemical composition of the biomass.

### Magnesium and potassium

Magnesium (Mg) and potassium (K) are important inorganic cations in the cells functioning, among other things, as cofactors of some enzymes. Mg is a cofactor of the kinase and synthetase enzymes, which are needed when acetate is converted to acetyl coenzyme A (CoA) entering the tricarboxylic acid cycle (Abbott et al. 1974; Fig. 3a in chapter 1.4.2). In *Acinetobacter* both Mg and Ca may function as counterions to the polyphosphate anions, and K seems to regulate the energetic state of the cell in the polyphosphate metabolism (van Groenestijn et al. 1988).

In spite of the potential importance of Ca in polyphosphate formation, this ion was not included in the optimization experiments, because there cannot be any shortage of Ca in the forest industry wastewater treatment. The concentrations of Ca are always very high (Table 9), since Ca is added to the wastewater as a neutralizing agent.

The responses of the three *Acinetobacter* isolates to different Mg and K levels were variable (Table 12, chapter 3.4). *A. lwoffii* had a clear optimum at  $6.0 \text{ mg l}^{-1}$  for both of the cations. *A. junii* showed an optimum at  $11.7 \text{ mg l}^{-1}$  Mg, but no optimum for K was found within the range of 2.0 to  $22 \text{ mg l}^{-1}$  K (Fig. 11a – b). *A. baumannii* did not show any response to either of these cations.

Since Mg and K are reported to be taken up and released simultaneously with phosphate (e.g. Mostert et al. 1988, Winter 1989), the requirement of poly-P bacteria for these ions is often calculated on the basis of released Mg:P and released K:P ratios.

The molar Mg:P ratios in the medium resulting in maximum growth of *A. lwoffii* and *A. junii* (0.93 and 1.8, respectively, calculated from Table 12) were high as compared with the reported molar ratios of released Mg to released P in sludge, where they range from 0.25 to 0.54 (Gerber et al. 1987a, Mostert et al. 1988, Toerien et al. 1990, Rickard and McClintock 1992). However, the amount of released Mg does not

necessarily reflect the amount of Mg bound in polyphosphates, nor does it reflect at all the Mg bound in other cellular components, and therefore it cannot be used to predict the Mg requirements of the poly-P bacteria. Some of the Mg associated with polyphosphates remains in the cell if only part of the polyphosphates is degraded. The estimates of Mg requirements based on the composition of the growth medium are more reliable than estimates based on Mg released.

The mode of polyphosphate accumulation (chapter 1.4.4) may affect the Mg requirements of bacteria. Dicks and Tempest (1966) and Dean and Rogers (1967) obtained a low molar Mg:P ratio of 0.12 for optimal growth of *Aerobacter aerogenes* in a chemostat culture. Roinestad and Yall (1970) observed maximum polyphosphate granulation in *Zoogloea ramigera* isolated from activated sludge at molar Mg:P ratios between 0.4 – 8.5. Excess phosphate uptake by these bacteria is due to the overplus phenomenon, while acinetobacters take up excess phosphate during growth.

The K concentration of  $6 \text{ mg l}^{-1}$  K, resulting in maximum growth of *A. lwoffii*, is a little lower than that obtained by van Groenestijn et al. (1988) for the maximum phosphate uptake by *Acinetobacter* 210A. For maximum phosphate uptake isolate 210A needed more than  $10 \text{ mg l}^{-1}$  K. In activated sludge maximum uptake rate was obtained with  $5 \text{ mg l}^{-1}$  K (van Groenestijn et al. 1988).

For *A. lwoffii* maximum growth was obtained with a ratio of C:N:P:K = 100:12:1.5:1.1 in the growth medium. According to Stanier et al. (1975), the average ratio (on a weight basis) of these elements in bacterial cells is 100:28:6:2. In relation to nitrogen, the K requirement of this isolate (K:N = 0.09) was close to the ratio (0.07) presented by Stanier et al. (1975). In relation to phosphorus, twice as much K (K:P = 0.7) was needed as predicted (0.3) on the basis of the ratio given by Stanier et al. (1975).

The molar K:P ratio (0.56) for *A. lwoffii* was almost the same as that obtained for optimal growth of *Aerobacter aerogenes* (0.5) in a chemostat culture (Dicks and Tempest 1966, Dean and Rogers 1967). This ratio was also within the range of the molar ratio of released K to P (from 0.38 to 0.64) in activated sludge studied by Mostert et al. (1988), but higher than the ratios (0.20 – 0.40) obtained in many other studies (a review by Toerien et al. 1990, Rickard and

McClintock 1992). According to van Groenestijn et al. (1988), uptake and release of K probably has no direct relationship to phosphate uptake and release, but instead, K release would take place when energy generation is limited (e.g. under anaerobic conditions). Thus the K:P ratios obtained in phosphate release experiments would not give reliable estimates of the K requirements of poly-P bacteria.

It is difficult to draw any conclusions on the different functions of K and Mg (polyphosphate metabolism versus other cellular functions) in a growing *Acinetobacter* culture. In *A. aerogenes* the molar Mg:K:P ratio tends to be constant (1:4:8) and independent of the growth rate, reflecting probably the composition of ribosomal structures (Tempest et al. 1966). In *A. lwoffii* the K:P ratio (molar Mg:K:P = 1:0.6:1.1) was close to the ratio of *A. aerogenes*. This ratio is probably common in bacteria. But the need for Mg in relation to P was much higher in acinetobacters than in *A. aerogenes* and possibly in many other bacteria as well. This supports the conclusion of van Groenestijn et al. (1988) that Mg, being a counterion, is directly coupled to polyphosphates, whereas K is coupled to polyphosphate metabolism indirectly. The high Mg requirements would then be due to polyphosphate formation during growth.

With the molar ratios of Mg:P = 1.8 and K:P = 0.56 and a phosphate concentration of 2 mg l<sup>-1</sup> P in the influent wastewater, the Mg and K requirements of acinetobacters would be 2.8 mg l<sup>-1</sup> Mg and 1.4 mg l<sup>-1</sup> K. Since the lowest Mg and K concentrations in the influents to the treatment plants were 2.5 mg l<sup>-1</sup> Mg and 6.3 mg l<sup>-1</sup> K (Table 9 in chapter 3.2), Mg or K would not be likely to limit the growth and concomitant polyphosphate formation of acinetobacters. There may, however, be strains such as *A. junii*, 910093, which would benefit from K concentrations higher than those found in wastewater. If Mg concentrations in the wastewater were too low, Mg as a counterion could probably be replaced by Ca, as found by van Groenestijn et al. (1988) for *Acinetobacter* isolate 210A. The Ca concentrations were very high in all treatment plants in relation to the needs of bacteria (Table 9).

In the treatment plants studied the spatial variations of Mg, Ca and K were relatively small. This may indicate that Mg, Ca and K were not actively involved in the metabolism of the sludge bacteria, or, in contrast, that their biological cycle

was very fast, or that their amounts clearly exceeded the need of sludge bacteria. Acinetobacters, which occurred in low concentrations in the sludge (chapter 3.3), could not have contributed to the cycles to any significant extent.

The reasons for the high Mg and K requirements of *A. junii*, isolate 910093, and the indifference of *A. baumannii* to these ions remain unexplained, but the results with the three isolates point to the difficulty of drawing any definite conclusions, except for rough generalizations, on the Mg and K requirements of acinetobacters.

### Optimal growth media

The results of the optimization experiments can also be used in designing an optimal growth medium for acinetobacters, as has been done in the present study of phosphate uptake kinetics.

The literature provides many different recipes for the acetate mineral medium (e.g. Fuhs and Chen 1975, Deinema et al. 1980, van Groenestijn 1988). Most authors do not give any comment on what basis their medium was compiled. However, as stated by Stanier et al. (1975) the design of a culture medium should be based on knowledge of the nutritional requirements of the bacteria.

Factorial experiments are a useful tool for optimization of growth media, but they have been seldom utilized (used e.g. by Brückner et al. 1991, Christen and Raimbault 1991).

### 4.3 Phosphate uptake kinetics of four *Acinetobacter* isolates and three other isolates

The ability of poly-P bacteria to take up phosphate, store excess phosphate rapidly and use polyphosphates as phosphorus and energy sources offers these organisms competitive advantage. This ability can be utilized in the treatment of both nutrient-deficient and nutrient-rich wastewaters. Forest industry wastewaters are phosphorus-limited in relation to carbon, especially if phosphorus is not added.

Excess phosphate uptake in bacteria may take place in three different ways (chapter 1.4.4): (1)

the overplus phenomenon, when the cells grown under phosphorus limitation are exposed to phosphorus (2) luxury uptake under unfavourable conditions after growth has ceased and (3) luxury uptake under favourable conditions during growth. The first and second types of excess uptake are found in many bacteria, but the third has been observed only in a few, including *Acinetobacter* (Fuhs and Chen 1975, Deinema et al. 1980, Hao and Chang 1987).

Phosphate uptake of *Acinetobacter* isolates through the overplus phenomenon is described by uptake model 1 (equation 9) and luxury uptake under favourable growth conditions by uptake models 2a – d (equations 10 – 17). Both models are analogous to the Michaelis-Menten model for enzyme kinetics (Michaelis and Menten 1913), to the Monod model for bacterial growth (Monod 1949) and to an adsorption isotherm. In phosphate uptake kinetics, biomass corresponds to the enzyme and phosphate to the substrate of the enzyme kinetic model.

The Michaelis-Menten equation is used commonly to describe saturation phenomena in various biological, chemical, pharmacological and medical processes in which some kind of an analogy to adsorption can be found (Droop 1968, Holmberg 1982). Phosphate uptake by planktonic bacteria and algae have also been found to follow Michaelis-Menten kinetics (e.g. Rhee 1973, Nyholm 1976, Cembella et al. 1984, Jansson 1993).

#### Chemical precipitation and physical adsorption of phosphate

In actively growing cultures of acinetobacters, pH values sometimes increased up to 7.7 in spite of good buffering. Above pH 7.5 calcium and magnesium may precipitate with phosphate (Arvin 1983, Miya et al. 1987). In alkaline conditions a precipitate of magnesium-ammonium phosphate may also be formed (Snoeyink and Jenkins 1980). Phosphate can also be adsorbed to the glassware. According to the control experiments, the amount of EDTA in the medium was sufficient to chelate the ions that would otherwise have precipitated under the experimental conditions. Thus the observed phosphate uptake was due to uptake by bacteria and not to any chemical or physical phenomena. Calculations of the solu-

bility product of magnesium-ammonium phosphate showed that this compound would not be precipitated in the phosphate uptake medium at any pH, whether there is EDTA present or not. Therefore the precipitate formed in the uptake medium without EDTA was most likely a magnesium or calcium salt of phosphate.

#### Phosphorus content of *Acinetobacter* biomass

The total phosphorus content of bacteria is of practical importance for the activated sludge process. When sludge accumulates large amounts of phosphorus per biomass unit, it removes phosphate without excessive sludge production. Minimizing sludge production is important, since it is difficult to dispose of the excess sludge that cannot be recycled in the process.

The total phosphorus content in *Acinetobacter* biomass is usually higher than in bacteria on an average. The phosphorus contents of acinetobacters are reported to vary from 2 to 10 %, depending on temperature, pH, growth rate and substrate limitations (Fuhs and Chen 1975, Deinema et al. 1985, van Groenestijn et al. 1989b, Hiraishi and Morishima 1990, Streichan et al. 1990, Appeldoorn et al. 1992a, Bonting et al. 1992a, b). There is, however, evidence that some other sludge bacteria (e.g. *Pseudomonas*) are as good as or even better accumulators of phosphate than acinetobacters, with phosphorus contents as high as 28 % (e.g. Hiraishi and Morishima 1990, Nakamura et al. 1991, Ubukata and Takii 1994).

The total phosphorus contents of the four *Acinetobacter* isolates (2.7 – 3.8 %, as estimated on the basis of phosphate taken up, Table 15 in chapter 3.5) grown in liquid acetate mineral medium containing 8 mg l<sup>-1</sup> P were within the lower range reported for acinetobacters in the literature. The phosphorus content analyzed in the biomass grown under phosphate limitation (about 1 %) was close to the phosphorus content of 1.3 % (estimated by phosphate uptake) reported by van Groenestijn et al. (1989b) for a phosphorus-limited *Acinetobacter* biomass.

In most experiments reported in the literature the initial phosphate concentrations have been much higher (up to 90 mg l<sup>-1</sup> P, van Groenestijn et al. 1989b) than in the present study (usually less than 10 mg l<sup>-1</sup> P). Hiraishi and Kitamura

(1984) observed that the phosphorus contents of a phototrophic *Rhodospseudomonas* species increased with increasing external phosphorus concentrations. The same has been found in the few studies with *Acinetobacter* where lower initial concentrations have also been used.

Phosphorus contents obtained by Hao and Chang (1987) and van Groenestijn et al. (1989b) at initial phosphate concentrations between 2.5 and 15 mg l<sup>-1</sup> P vary from 1 to 4 %, which is practically the same as obtained in the present study. For instance the phosphorus content analyzed in an *Acinetobacter* isolate grown in a chemostat by Hao and Chang (1987) was 1 % at 2.5 mg l<sup>-1</sup> P, 2 % at 5 mg l<sup>-1</sup> P, 2–3 % at 10 mg l<sup>-1</sup> P (a concentration comparable with this study) and 3–5 % at 34 mg l<sup>-1</sup> P.

The maximum phosphorus contents reported for activated sludge with enhanced phosphorus removal typically vary from 3.5 to 6.8 % in full-scale municipal plants and from 11 to 18 % in pilot- or laboratory-scale experiments with synthetic wastewater (reviewed by Appeldoorn et al. 1992b). The difference between the full-scale plants and pilot- and laboratory-scale experiments is due to the lower concentrations of volatile fatty acids in the real than in the synthetic wastewater.

In microorganisms the difference between the phosphorus content of a cell which has utilized its internal phosphorus reserve after growing under phosphorus-limited conditions and a cell grown in non-limiting phosphorus concentrations, may be more than 10-fold (Nyholm 1976). For instance, in a planktonic alga *Selenastrum capricornutum* and a cyanobacterium *Nostoc* sp. the phosphorus content of the cells varied from 0.1 to 2.5 % P per dry cell mass (Brown and Harris 1978). In comparison with those figures, the observed 3- to 4-fold variation in the phosphorus content of an *Acinetobacter* isolate was small.

The varying abilities of *Acinetobacter* isolates to accumulate phosphate may reflect the different origins of these bacteria. *Acinetobacters* originating from municipal sludge are adapted to higher phosphorus levels than *acinetobacters* from sludge treating forest industry wastewaters. In a phosphate-rich environment the ability to utilize low phosphate concentrations is not crucial. Judging by the studies of van Groenestijn et al. (1989b) and Bonting et al. (1992b),

different culture conditions (chemostat or batch) or carbon sources (butyrate or acetate etc.) would not have any major effect on the phosphorus content.

In contrast with the results of the present study, Bonting et al. (1992a) and Hiraishi and Morishima (1990) could not detect any polyphosphate granules after Neisser staining in biomass with phosphorus contents of 1.5 and 2.7 %, respectively. In the present study the cells with a phosphorus content of 2.7 % showed clearly visible polyphosphate granules during growth (c.f. Fig. 13 in chapter 3.5 for cells with a phosphorus content of 3.5 % after 18 h). About 75–100 % of the cells contained granules. In some cases the granules filled the whole cell so that the cell looked completely dark under the microscope. It is unlikely that dark granules of this kind would have been anything else but polyphosphate granules.

It seems that the total phosphorus analysis for water or wastewater underestimates of the total phosphorus content of biomass containing polyphosphates. The amount of phosphorus analyzed was on an average 46 % of that estimated on the basis of phosphate uptake data. It seems that these methods do not degrade all polyphosphate fractions into phosphate (methodological difficulties of polyphosphate analysis are discussed by Kulaev and Vagabov 1983). The total phosphorus content analyzed in biomass devoid of polyphosphates (time 0 h) may, however, be correct.

Apparently, a small part of polyphosphates was degraded with the peroxodisulphate treatment, since the analyzed values at time 0 h were lower than at the end of the experiment (Table 15). It is also possible that the cells excrete organic phosphorus compounds during growth (Shoda et al. 1980), and thus the total phosphorus analysis correctly gives the net phosphorus uptake, while the figures based on phosphate consumed in the medium represent the gross uptake.

The results of different studies are not always directly comparable, since there are differences in the methods used in the various studies. Some authors have analyzed total phosphorus concentrations with the peroxodisulphate or some other method, and others have estimated them on the basis of phosphate uptake. Rough comparisons can, however, be made.

### The overplus phenomenon (uptake model 1)

The so-called transient phosphate uptake rate (i.e. rate following exposure of a phosphorus-limited biomass to phosphate) of a microorganism may be controlled by the phosphorus content of the cell (Rosenberg et al. 1969, Brown and Harris 1978, Cembella et al. 1984). In poly-P bacteria devoid of polyphosphate storage, growth does not necessarily take place immediately after the phosphorus addition, but phosphate is first stored by the cells. This phenomenon was also observed in the short-term batch experiments, when the acinetobacters grown in the phosphorus-limited medium were suspended in the phosphate uptake medium. No growth took place during the initial, rapid phosphate uptake. This does not, however, seem to be the case with all acinetobacters and under all conditions. For instance in the chemostat culture of *A. johnsonii* 210A studied by Bonting et al. (1992a), addition of phosphate to the starved cells resulted in biomass formation. Polyphosphate was accumulated only after maximum growth of the biomass was attained.

The initial phosphate uptake rate of a phosphate-starved *Acinetobacter* biomass followed the Michaelis-Menten (or Monod) kinetics (Fig. 12). In this type of model the initial uptake rate ( $v_0$ ) increases with increasing substrate concentrations until further substrate additions no longer have an effect on the uptake rate, i.e. the maximum initial uptake rate ( $V_{max}$ ) is achieved (Clark and Switzer 1977).

Bacteria with the steepest slope of the uptake curve in the Michaelis-Menten plot have competitive advantage over other bacteria in a nutrient-deficient environment (Healey 1980). The kinetic parameters were estimated on the basis of the pooled data of the three *Acinetobacter* isolates. There were, however, slight differences between the initial slopes of the uptake curves of the isolates, although due to measurement noise their significance is questionable. The linear slopes at concentrations below 10 mg l<sup>-1</sup> P would seem to indicate that *A. junii*, 910093 is the most efficient (slope 1.3) in excess phosphate uptake and *A. baumannii*, 910045 the least efficient (slope 0.83).

The initial phosphate uptake rates of phosphate-starved biomass could be used to calculate uptake when the return sludge containing starved cells is mixed with the influent wastewater. These

rates could also be used to estimate the phosphate uptake of cells entering an aerobic phase after being exposed to anaerobic conditions in treatment plants with aerobic and anaerobic sequences. According to the biochemical models (chapter 1.4.2), the cells degrade their polyphosphate storage during anaerobiosis, but, like the phosphate-starved *Acinetobacter* biomass after cultivation in the phosphorus-limited medium, they contain a carbon storage. The carbon storage is utilized as a carbon and energy source, which enables rapid phosphate uptake.

The so-called half-saturation constants,  $K_M$  (uptake model 1) and  $K_s$  (uptake models 2a – d), give the phosphate concentrations which sustain half of the maximum phosphate uptake rate and half of the specific growth rate, respectively. In planktonic algae  $K_M$  for uptake may be 10 times as high as  $K_s$  for growth (Rhee 1973, Cembella et al. 1984). The average  $K_M$  of the *Acinetobacter* isolates for excess phosphate uptake (17 mg l<sup>-1</sup> P) was about five times the average  $K_s$  for growth (3.2 mg l<sup>-1</sup> P).

The  $K_M$  value of 17 mg l<sup>-1</sup> P given by uptake model 1 indicates that the ability of the studied acinetobacters to take up excess phosphate through the overplus phenomenon is best with phosphate concentrations much higher than those found in forest industry wastewaters or even in municipal wastewaters. Whether the  $K_M$  value is high or low for excess phosphate uptake in general is difficult to evaluate, since the half-saturation constants presented in the literature are related to growth, and not to phosphate uptake.

The maximum initial uptake rate of excess phosphate (29 mg P g<sup>-1</sup> DM h<sup>-1</sup>) is approached only when the phosphorus concentration reaches several hundred milligrams per litre, i.e. a concentration which does not occur in wastewater treatment. In corresponding phosphate uptake experiments with *Acinetobacter* 210A by van Groenestijn et al. (1989b), the initial uptake rate of 29 mg P g<sup>-1</sup> DM h<sup>-1</sup> was obtained at a much lower initial phosphate concentration, namely 15 mg l<sup>-1</sup> P. These authors do not present any data on the uptake rates at other concentrations, but this rate hardly represents the maximum uptake rate of the isolate.

The phosphorus removal capacity of acinetobacters was close to the capacity obtained by Ubukata and Takii (1994) for an unidentified

coccoid bacterium isolated in activated sludge. With an initial phosphate concentration of  $60 \text{ mg l}^{-1} \text{ P}$ , this isolate was able to take up  $20 \text{ mg P g}^{-1} \text{ DM h}^{-1}$  after being exposed to alternating aerobic and anaerobic conditions. The corresponding uptake rate calculated with model 1 for the phosphorus-starved acinetobacter biomass in the present study would be  $23 \text{ mg P g}^{-1} \text{ DM h}^{-1}$ .

Part of the variation in the uptake data (Fig. 12) is due to the varying ratios of the initial phosphate concentration to the biomass in the individual uptake experiments. The same initial phosphate concentration ( $P_0$ ) results in different uptake rates per biomass unit depending on the biomass (B): for a given phosphate concentration, a high biomass concentration results in a higher uptake rate per biomass unit than a low biomass concentration. This is obvious since the ratio  $P_0:B$  is actually the cell phosphorus quota, which is inversely proportional to the uptake rate (Brown and Harris 1978). It takes longer for the cell phosphorus quota to increase to a point where the uptake rate begins to decrease with high than with low biomass concentrations. Nevertheless the rate of phosphate uptake into the cell may be the same at the onset of the uptake process.

In determining the dependence of the initial phosphate uptake rate on phosphate concentration, the  $P_0:B$  ratio should be kept as constant as possible in the experiments. This ratio should be neither too low nor too high. If the biomass concentration is high relative to the phosphate concentration, the initial linear uptake occurs within a very limited time interval and is difficult to determine. Again if the biomass concentration is low, the uptake rate is slow as well, and has to be followed for a long time, which may in turn result in some growth. In the present study the biomass concentrations were about the same as or slightly lower than those estimated for the living biomass in activated sludge in the aeration basin (assuming on the basis of the ignition loss of sludge that half of the sludge dry mass is microbial).

The initial adsorption of phosphate to the cell surface may also have a measurable influence on the uptake rate. A big biomass has large surface where phosphate is bound before being absorbed into the cell. Thus, phosphate may disappear from the growth medium more rapidly with a high than with a low biomass concentration.

Only one of the three non-acinetobacters

isolated on the acetate mineral medium (*Agrobacter radiobacter*, isolate 920012) showed excess phosphate uptake by the overplus phenomenon. The uptake rate was about one fifth of the corresponding rate calculated from uptake model 1 for acinetobacters.

The Neisser stain is a useful screening method of the ability of the cells to accumulate polyphosphates, but it does not reflect the efficiency of phosphate uptake. Some of the cells of the non-acinetobacter isolates contained granules with less intense violet colour than *Acinetobacter* cells, but their excess phosphate uptake was slight or could not be distinguished from phosphate uptake for growth.

The present phosphate uptake experiments relate only to acinetobacters and other bacteria favouring acetate as a carbon source. In order to study the phosphate uptake kinetics of other poly-P bacteria other types of media should be developed in which the phosphate concentration can be controlled.

#### Phosphate uptake kinetics under favourable growth conditions (uptake model 2)

These experiments were performed in a medium optimal for the growth of acinetobacters (chapter 2.8). Since the Neisser stained cells contained polyphosphate granules during the exponential growth phase and not only during the stationary phase, phosphate uptake was due to "luxury uptake of phosphate under favourable conditions" (chapter 1.4.4). *Acinetobacter* is one of the few bacteria known to have this type of excess uptake (Fuhs and Chen 1975, Deinema et al. 1980, Hao and Chang 1987). It is more common in planktonic algae (Cembella et al. 1984).

The Michaelis-Menten equation describes adequately the relationship between substrate uptake and growth if the substrate is metabolized soon after its assimilation. In polyphosphate accumulating microorganisms growth and uptake rates may also depend on the amount of phosphorus stored in the cells, (i.e. the phosphorus content of the cell or the cell phosphorus quota), and a direct relationship does not necessarily exist between the growth and uptake rates and the concentration of the extracellular phosphate (Fuhs 1969, Rhee 1973, Nyholm 1976).

An inverse relationship between the phosphate

uptake rate and the cell phosphorus quota has been observed for algae by Brown and Harris (1978). With an increasing cell phosphorus quota the phosphate uptake rate decreases approaching zero. With the utilization of the internal phosphorus storage for growth, uptake in the medium again increases. In this case the Droop equation (Droop 1968), relating the specific growth rate to the difference between the actual cellular phosphorus concentration and the minimum concentration required for growth, can be applied.

In the present study the phosphate storage formed in the *Acinetobacter* cells (less than 4 % P in dry biomass) was apparently not large enough to induce utilization of the intracellular polyphosphate pool in such a magnitude that a reduction in phosphate uptake rate would have been observed. The Michaelis-Menten model described the relationship between growth and phosphate uptake without significant deviations from the measured values.

It is also possible that the intracellular pool actually had an effect on the overall uptake process, but this could not be observed with the present sampling scheme with at least one hour's intervals. It can be hypothesized that a more frequent sampling, for instance every 30 minutes or even more often, would have revealed a cyclic pattern of uptake: first a rapid uptake of phosphate per cell from the medium without growth, followed by growth at the expense of the intracellular phosphorus without phosphate uptake from the medium etc. (presented schematically in Fig. 16; c.f. Fig. 14a - d in chapter 3.5). This kind of pattern could, however, be realized only if the growth of bacteria were synchronized. This is unlikely after a couple of bacterial generations.

Utilization of polyphosphates was observed in *Acinetobacter junii*, isolate 920121, at the end of the experiment (Fig. 14d). The phosphate concentration in the medium had dropped to zero in less than 19 hours, but the biomass continued to grow with a high rate. Utilization of polyphosphates was also observed under the microscope. After 18 and 18.5 h incubation periods all Neisser stained cells had dark polyphosphate granules (Fig. 13), and after 19 and 19.5 h all cells still contained granules, but they were lighter than an hour earlier. After 20 h the cells were almost devoid of granules.

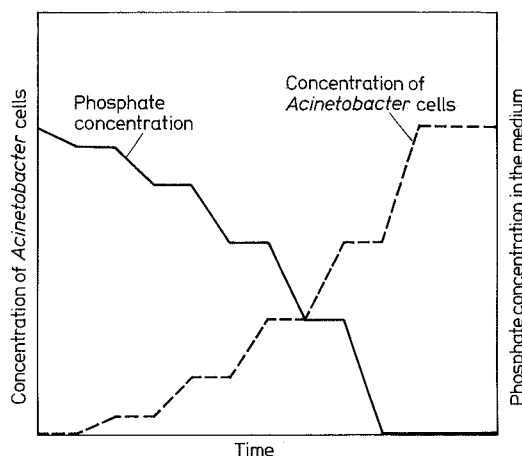


Fig. 16. A schematic presentation of the relationship between growth and phosphate uptake of a polyphosphate-storing microorganism: phosphate uptake and storage without growth followed by growth at the expense of the intracellular phosphorus without phosphate uptake from the medium etc.

The rapid utilization of the internal phosphorus storage by *Acinetobacter* is in accordance with observations on other microorganisms. Growth rates of bacteria and algae have been found to be linear functions of the intracellular phosphorus storage after exhaustion of phosphate in the medium (Slezák and Sikyta 1967, Rhee 1973, Nyholm 1976, Cembella et al. 1984).

After exhaustion of phosphate in the medium, the Michaelis-Menten model (model 2d) cannot describe the phenomenon any further. The model predicts erroneously a slight increase in the phosphate concentration in the medium (cannot be observed on the scale of Fig. 14d).

The growth of the other three *Acinetobacter* isolates seemed to level off at the same time as the phosphate in the medium was exhausted. Isolates 910045 and 910093 originated from enrichment cultures of activated sludge, and during the enrichment process they probably lost some of their original characteristics, such as the ability to grow by using internal polyphosphate storage as a phosphorus source. *A. lwoffii*, 9003 had been isolated in activated sludge fed with phosphate, and probably it had not had a need for a mechanism for immediate utilization of the internal polyphosphate storage. *A. junii*, 920121, originated from influent wastewater with a low



phosphate concentration, where the ability to store phosphate rapidly and utilize the storage provides significant competitive advantage.

The parameters  $\mu_{\max}$  and  $K_s$  are sensitive to measurement noise (Holmberg 1982). The accuracy of determination of these parameters depends among other things on the ratio  $K_s:P_o$ . Holmberg (1982) has shown that for  $K_s:P_o$  ratios lower than 4 the estimates obtained with the Michaelis-Menten equation are more accurate than those obtained with a linear model. For acinetobacters  $K_s:P_o$  varied from 0.02 to 0.75, justifying the use of the Michaelis-Menten equation in this respect. In theory, if the  $K_s:P_o$  ratio is lower than 0.1, the estimate of  $\mu_{\max}$  is exact, provided the measurements are not very noisy, whereas the deviation of  $K_s$  is significant (Holmberg 1982).

The maximum specific growth rates ( $\mu_{\max}$ ) of the isolates (from 0.15 h<sup>-1</sup> for *A. junii*, 920121, to 0.55 h<sup>-1</sup> for *A. lwoffii*, 9003, Table 13) were somewhat lower than those obtained for acinetobacters isolated in municipal sludge (0.42 and 0.83 h<sup>-1</sup> in ethanol and acetate, respectively, Hao and Chang 1987; and 0.69 h<sup>-1</sup> in acetate, van Groenestijn et al. 1989b) or in soil (0.7 h<sup>-1</sup> in ethanol, Abbot et al. 1973). Nevertheless, the rates were clearly higher than those obtained for a phosphorus-accumulating *Micrococcus* (0.05 h<sup>-1</sup>) in an enrichment culture of activated sludge (Nakamura et al. 1991), or for *Acinetobacter* and *Pseudomonas* isolates (0.03 h<sup>-1</sup>) in an acetate-fed laboratory-scale reactor (Okada et al. 1992).

A low growth rate may be a useful property in sludge bacteria if the phosphate uptake rate per biomass unit is high: less sludge is formed per phosphorus removed. In the study of Okada et al. (1992) isolates with the highest phosphorus removal activities had the lowest specific growth rates. However, it seems likely that bacteria with very low growth rates cannot compete efficiently for substrates with other bacteria in the same environment. The reciprocal of the specific growth rate has to be lower than the sludge retention time in order that the bacteria are not washed out of the treatment plant. Since the retention times of sludge are several days, *Acinetobacter* biomass with the specific growth rates obtained in the present study would not be washed out.

*A. lwoffii*, 9003, had the highest maximum phosphate uptake rate ( $V_{\max} = 12$  mg P g<sup>-1</sup> DM

h<sup>-1</sup>) and *A. junii*, 920121, the lowest (3.7 mg P g<sup>-1</sup> DM h<sup>-1</sup>). In the influent of pulp mill 2 (the origin of isolate 920121) a higher uptake rate would not be needed in any case, since the phosphate concentrations were very low. More important was the ability of isolate 920121 to utilize efficiently low phosphate concentrations, as will be discussed later in this chapter.

In bacterial growth kinetics  $K_s$  values are often considered as indicators of the affinity of bacteria for the substrate: a low  $K_s$  reflects an efficient uptake system for the substrate. The  $K_s$  (0.40 mg l<sup>-1</sup> P) of *A. junii*, isolate 920121, was an order of magnitude lower than the  $K_s$  values of the other isolates (Table 13 in chapter 3.5), indicating good adaptation of isolate 920121 to growing at low phosphate concentrations.

Healey (1980) has criticized the widespread use of the  $K_s$  value as a sole measure of the competitiveness of a microorganism at low nutrient concentrations, since  $K_s$  may vary with  $\mu_{\max}$  and  $V_{\max}$ . Bacteria with low  $K_s$  values do not attain high maximum growth or uptake rates, which was also observed for *A. junii*, isolate 920121.

Healey (1980) and Lobry et al. (1992) have suggested that the ratios  $\mu_{\max}/K_s$  and  $V_{\max}/K_s$  could be used as indicators of the ability of bacteria to compete for the substrate at low concentrations. The higher the ratio  $\mu_{\max}/K_s$ , the faster the bacteria grow at low concentrations of the limiting nutrient, and the higher the ratio  $V_{\max}/K_s$ , the more efficient they are in utilizing low nutrient concentrations. These ratios are actually the slopes of the corresponding Michaelis-Menten (or Monod) equations at the lowest substrate concentrations (Healey 1980).

In models 2a – c the ratios  $\mu_{\max}/K_s$  (Table 14 in chapter 3.5) were fairly close to each other, varying from 0.080 l mg<sup>-1</sup> h<sup>-1</sup> P (*A. junii*, 910093, model 2c) to 0.092 l mg<sup>-1</sup> h<sup>-1</sup> P (*A. lwoffii*, 9003, model 2a). This is clearly exemplified by a plot of  $K_s$  and  $\mu_{\max}$ , which shows a very good positive linear correlation between the values of  $K_s$  and  $\mu_{\max}$  obtained by models 2a – c ( $\mu_{\max} = 0.10K_s - 0.05$ ,  $r = 1.00$ , Fig. 17). The linear slope does not, however, run exactly through the origo, as it theoretically should (Lobry et al. 1992). In model 2d (*A. junii*, 920121) the ratio  $\mu_{\max}/K_s$  was about 4 – fold (0.375 l mg<sup>-1</sup> h<sup>-1</sup> P) as compared with the ratios of the other isolates. This can also be observed in

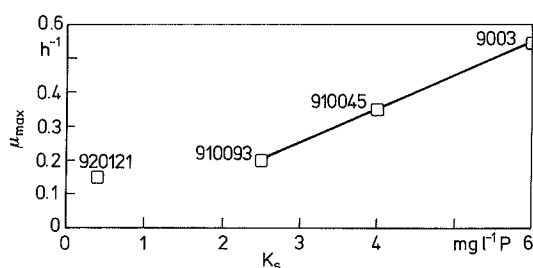


Fig. 17. The relationship between the maximum specific growth rate ( $\mu_{max}$ ) and the half-saturation constant ( $K_s$ ) for *Acinetobacter lwoffii*, isolate 9003, *Acinetobacter baumannii*, isolate 910045, and *Acinetobacter junii*, isolates 910093 and 920121. For isolates 9003, 910045 and 910093  $\mu_{max} = 0.10K_s - 0.05$  ( $r = 1.00$ ).

Fig. 17. In this plot the ratios for bacteria with more efficient uptake systems than those of isolates 9003, 910045 and 910093 would be above the line and *vice versa*.

The ratio  $V_{max}/K_s$  varied from 1.4 to 2.3 l g<sup>-1</sup> DM h<sup>-1</sup> in models 2a – c, being almost 5 – fold (9.3 l g<sup>-1</sup> DM h<sup>-1</sup>) in model 2d. It is interesting to note that the  $V_{max}/K_M$  ratio by model 1 describing excess phosphate uptake due to the overplus phenomenon is almost the same (1.7 l g<sup>-1</sup> DM h<sup>-1</sup>) as the average  $V_{max}/K_s$  ratio (1.9 l g<sup>-1</sup> DM h<sup>-1</sup>) by models 2a – c (for the same isolates *A. lwoffii*, 9003, *A. baumannii*, 910045, and *A. junii*, 910093 as in model 1).

The main conclusion that can be drawn on the basis of the ratios  $\mu_{max}/K_s$  and  $V_{max}/K_s$  is almost the same as that drawn on the basis of the  $K_s$  values alone. According to the  $K_s$  values there would be some differences in the affinities of isolates *A. lwoffii*, 9003, *A. baumannii*, 910045, and *A. junii*, 910093, for phosphate (Table 13, chapter 3.5). Based on the ratios, however, none of these three isolates would have significant competitive advantage over the other two at low phosphate concentrations, at least not under the experimental conditions used. Instead, *A. junii*, isolate 920121 would be very competitive in a phosphate-deficient environment in relation to the other three isolates – and probably in relation to many other bacteria as well. This could be concluded on the basis of the  $K_s$  values as well.

The different affinities of the two *A. junii* isolates (910093 and 920121) for phosphate support the views of Beacham et al. (1992) and Bosch and Cloete (1993) that the ability of

acinetobacters to accumulate polyphosphate is not dependent on their taxonomic status, i.e. species.

The different origins of the four *Acinetobacter* isolates may explain differences in their phosphate uptake kinetics, as discussed earlier in this chapter.

The most efficient of the acinetobacters studied, *A. junii*, 920121, was isolated in the phosphate-poor influent wastewater (1 mg l<sup>-1</sup> P) to the treatment plant of pulp mill 2. Bacteria living in this kind of environment can be expected to have efficient uptake systems for phosphate. On the basis of the  $\mu_{max}/K_s$  ratios the efficiency of *A. lwoffii*, 9003, isolated in the return sludge of the treatment plant of paper mill 1 during a period with relatively high phosphate additions, was about the same as the efficiency of the isolates (*A. baumannii*, 910045, and *A. junii*, 910093) originating from the enrichment cultures of activated sludge from pulp mill 1. Based on the low phosphate concentration in the influent to the treatment plant of pulp mill 1 (about 1 mg l<sup>-1</sup> P), more efficient phosphate uptake by acinetobacters would have been expected. The enrichment procedure (5 mg l<sup>-1</sup> P in the medium) had probably selected for less efficient strains or affected the uptake systems of these isolates.

Because the phosphorus level in forest industry wastewaters is fairly low, the phosphate uptake system of bacteria treating this kind of wastewater may be more efficient than that of bacteria in municipal treatment plants. However, as compared with bacteria isolated in nutrient deficient natural waters, the affinities of any sludge bacteria for phosphate are low. For instance the  $K_s$  value obtained for a *Pseudomonas* isolate from the Bothnian Sea was 0.095 mg l<sup>-1</sup> P (Jansson 1993). The  $V_{max}$  value for this *Pseudomonas* was of the same order of magnitude as for acinetobacters (10 mg P g<sup>-1</sup> DM h<sup>-1</sup>; the rate given per mg carbon is converted to dry mass by assuming a ratio of C/DM = 0.5, Sorokin and Kadota 1972). The ratio  $V_{max}/K_s$  of 110.5 for this marine *Pseudomonas* clearly shows the superiority of the phosphate uptake system of the pelagic bacterium, as compared with the ratio (9.3) obtained for the most efficient of the *Acinetobacter* isolates of this study (Table 14). Of course, comparisons between results obtained with different methods and culture conditions should

be made cautiously, since they may lead to erroneous conclusions.

In the present study it was found that the phosphate uptake rate of an *Acinetobacter* isolate subcultured for many months was lower than that of the same isolate some months before (this data was not included in the models). This may be due to changes in the physiological condition of the bacteria (Nyholm 1976) or, perhaps even more likely, due to genetic changes that had taken place during subculturing.

To minimize variation caused by the physiological or genetic changes, only stock cultures or recent subcultures of the stock should be used for inoculation. Successive transfers of bacteria are, however, a common way of storing bacteria (e.g. van Groenestijn et al. 1987, Hao and Chang 1987) which are frequently used as inocula. For instance van Groenestijn et al. (1987) maintained *Acinetobacter* isolate 210A on yeast extract medium, subcultured the isolate every second month and stored it at 4 °C. It may be questioned whether this practice has had an effect on the phosphate uptake rates reported in the literature.

It has to be emphasized that results of pure cultures in an artificial medium cannot be used as such to predict the behaviour of a microorganism in natural mixed populations and in complex, varying media such as wastewater. However, pure culture studies may give an indication of what is to be expected and thus form a basis for further studies with increasing complexity (mixed pure cultures or natural communities; complex media or natural environment etc.). Knowledge of the behaviour of a microorganism under controlled conditions may help to understand the phenomenon and to distinguish between various effects under natural conditions. As stated by Gest (1993) the study of pure cultures remains as the most reliable source of basic information for understanding the properties of bacteria.

#### Phosphate uptake potential of *Acinetobacter* populations in activated sludge

With low phosphorus concentrations typical of the influents to the treatment plants of pulp and paper mills where the total phosphorus concentration is often below 4 mg l<sup>-1</sup> and phosphate-P concentration varies from 1 to 2 mg

l<sup>-1</sup>, uptake models 1 and 2 give nearly the same results. For instance with an initial phosphate concentration of 2 mg l<sup>-1</sup> P, the phosphate uptake rates would be 3.1 mg P g<sup>-1</sup> DM h<sup>-1</sup> (model 1) and 2.6 mg P g<sup>-1</sup> DM h<sup>-1</sup> (average of models 2a – d). With increasing initial phosphate concentrations the differences between the uptake rates obtained by the two models grow more marked. This is to be expected, since phosphate uptake by a phosphorus-starved biomass (the overplus phenomenon) is a very rapid process, while luxury uptake by a growing biomass depends on the growth rate of the bacteria as well. In sludge both types of excess phosphate uptake may occur. The overplus phenomenon probably dominates at the very beginning of the aeration phase, and luxury uptake later in the process (for instance in the uptake of phosphate formed *in situ* in the degradation of organic matter).

The phosphate uptake rates of the *Acinetobacter* isolates were almost ten times the rates reported by Ye et al. (1988) for their *Acinetobacter* isolate (0.53 – 0.86 mg P g<sup>-1</sup> DM h<sup>-1</sup> at an initial phosphate concentration of 12 mg l<sup>-1</sup> P). Calculated by the uptake models 2a – d the corresponding uptake rates for the four *Acinetobacter* isolates would be between 3.6 and 7.9 mg P g<sup>-1</sup> DM h<sup>-1</sup>. These rates are comparable with the rates obtained for some unidentified sludge bacteria, and higher than those obtained for *Pseudomonas aeruginosa* (2.0 mg P g<sup>-1</sup> DM h<sup>-1</sup>) by Ye et al. (1988).

Phosphate uptake rates of sludge obtained by Winter (1989) in a laboratory-scale Bardenpho process treating municipal wastewater and fed with 20 mg l<sup>-1</sup> P and sodium acetate (60 mg l<sup>-1</sup> C) varied from 3 to 11 mg P g<sup>-1</sup> DM h<sup>-1</sup> (assuming that 50 % of the dry sludge was microbial biomass). These uptake rates are of the same order of magnitude as the rates calculated for the *Acinetobacter* isolates at the same initial phosphate concentration (3.6 – 9.1 mg P g<sup>-1</sup> DM h<sup>-1</sup>, models 2a – d). On the basis of the high pH values and changes in the chemical composition of the mixed liquor, Winter (1989) assumed that part of the observed good phosphate removal (95 %) by this sludge was due to chemical precipitation. However, on the basis of the phosphate uptake capacity of acinetobacters, sludge enriched with poly-P bacteria could also be responsible for this kind of removal.

For efficient phosphate uptake a sufficient

number of polyphosphate bacteria is also needed. For instance at a phosphate concentration of  $2 \text{ mg l}^{-1} \text{ P}$  and with an acinetobacter concentration of  $110 \text{ mg DM l}^{-1}$  or  $1 \cdot 10^8 \text{ cells ml}^{-1}$  the phosphate uptake rate would be  $8 \text{ g m}^{-3} \text{ d}^{-1} \text{ P}$  (model 1).

Two examples are given below on the size of the acinetobacter population needed to remove the daily phosphorus load of paper mill 1 and pulp mill 2. It is assumed that phosphate uptake follows models 1 and 2a – d and that acinetobacter biomass is evenly distributed in the aeration basin.

The phosphorus load from paper mill 1 was  $45 \text{ kg d}^{-1} \text{ P}$  during the study period, and the concentration of phosphate after the phosphoric acid addition  $4 \text{ mg l}^{-1} \text{ P}$ . The average wastewater flow was  $11\,100 \text{ m}^3 \text{ d}^{-1}$ . At this phosphate concentration the uptake rate would be  $5 \text{ mg P g}^{-1} \text{ DM h}^{-1}$ , which is an average given by model 1 and model 2a (obtained for *A. lwoffii*, 9003, isolated from the return sludge of this mill). Based on these figures and the volume of the aeration basin it can be calculated that an acinetobacter biomass of  $370 \text{ kg DM d}^{-1}$ , corresponding to an acinetobacter concentration of  $3 \cdot 10^7 \text{ cells ml}^{-1}$  in the aeration basin, would be needed to remove the daily phosphorus load.

*Acinetobacter* concentration in the sludge of this treatment plant was of the order of  $10^5 - 10^6 \text{ cells ml}^{-1}$  at the most, which means that acinetobacters could not account for more than a few per cent of the removal of the phosphorus load in paper mill 1. This percentage is about the same as the percentage of acinetobacters in the total cultivable population of sludge bacteria (chapter 3.3).

In theory, the volatile fatty acids in the influent of paper mill 1 ( $170 \text{ mg l}^{-1} \text{ C}$ ) could support an *Acinetobacter* population of  $2 \cdot 10^8 \text{ cells ml}^{-1}$ , which could remove all phosphorus in the wastewater. In practice, of course, other bacteria, e.g. denitrifiers, would also assimilate volatile fatty acids. However, this calculation indicates that if the treatment plants were built or modified and operated so as to favour the enrichment of acinetobacters (anaerobic and aerobic phases), enhanced biological phosphorus removal might well be accomplished. According to the optimization experiments (chapters 3.4 and 4.2) there would also be enough of inorganic ions essential for polyphosphate metabolism in the

wastewater.

The phosphorus load from pulp mill 2 was  $170 \text{ kg d}^{-1} \text{ P}$ , and the concentration of phosphate  $1.0 \text{ mg l}^{-1} \text{ P}$  with no added phosphorus. The average wastewater flow was  $111\,500 \text{ m}^3 \text{ d}^{-1}$ . At this phosphate concentration the uptake rate would be  $2.7 \text{ mg P g}^{-1} \text{ DM h}^{-1}$ , given by model 2d for *A. junii*, 920121, which formed nine out of ten isolates on the acetate mineral medium spread with the influent from this mill. Based on these figures and the volume of the aeration basin, an *Acinetobacter* biomass of  $2\,600 \text{ kg DM d}^{-1}$ , corresponding to an *Acinetobacter* concentration of  $7 \cdot 10^7 \text{ cells ml}^{-1}$  in the aeration basin, would remove the daily phosphorus load.

Assuming that the *Acinetobacter* concentration in the sludge (i.e. mixed liquor) is about the same as in the influent wastewater ( $1.3 \cdot 10^5 \text{ cells ml}^{-1}$ ), only 0.2 % of the phosphorus load would be removed by these bacteria.

The phosphorus removal of over 70 % occasionally observed in this treatment plant (Lammi and Pakarinen 1993) could have been due to the normal metabolism of heterotrophic bacteria, to the overplus phenomenon when the return sludge is mixed with the influent, and to other poly-P bacteria, as well as to adsorption and chemical precipitation on the flocs. It is possible that the remaining 30 % of the phosphorus load could be removed by favouring the growth of the relatively efficient *Acinetobacter* population.

Judging by these two examples, acinetobacters did not play a significant role in the phosphorus removal in these aerobic treatment processes. In spite of the possible *Acinetobacter* inocula in the influent wastewater, the *Acinetobacter* population in the activated sludge was not large enough to be detected among the most common bacteria. This was not an unexpected result in aerobic treatment plants.

However, especially in the case of paper mills there are reasons to believe that biological phosphorus removal could be achieved by simply arranging aerobic and anaerobic phases so as to enrich acinetobacters (and other poly-P bacteria with similar metabolic traits) and select for acinetobacters against other strictly aerobic bacteria. Pulp mill wastewaters may not contain enough of suitable carbon sources (analysis made only in the treatment plant of pulp mill 1). However, these compounds could probably be generated by fermentation. This would be an

important subject for further studies.

The phosphorus removal capacity of the *Acinetobacter* population estimated for the aerobic forest industry treatment plants is lower than the average capacities calculated by Cloete et al. (1985) and Cloete and Stayn (1988b) for sludge treating municipal wastewaters in alternating aerobic and anaerobic conditions. Their estimates vary from 3 to 34 % of the observed phosphorus removal, averaging 10 % (Cloete et al. 1985) and 17 % (Cloete and Stayn 1988b).

#### 4.4 Phosphate release kinetics of five *Acinetobacter* isolates

In general, the ability of sludge to release phosphate under anaerobic conditions is regarded as an indirect indication of a well-functioning phosphorus removal process (e.g. Fuhs and Chen 1975, Abu-gharah and Randall 1991, Appeldoorn et al. 1992b). This seems logical, since cells still possessing polyphosphates stored during the previous aerobic phase could not take up additional phosphate. Phosphate release – if not due to cell autolysis only – is also an indication of the presence of poly-P bacteria. According to Abu-gharah and Randall (1991) the amount of phosphate released can even be used to predict phosphate uptake. However, a relationship between the phosphate release and the phosphorus removal capacity of sludge has not always been observed (Daigger et al. 1987).

The linearity of phosphate release with respect to time observed for the *Acinetobacter* isolates has also been found in activated sludge (Hashimoto and Furukawa 1984).

Phosphate release was different in the five *Acinetobacter* isolates studied (variation from 0 to  $0.26 \text{ mg P g}^{-1} \text{ DM h}^{-1}$ , Table 16, Fig. 15a – d). For instance *A. junii*, isolate 920121, released much more phosphate than *A. lwoffii*, isolate 920122, which originated from the same sample of influent wastewater.

The release rates obtained for the *Acinetobacter* isolates were close to the rate of  $0.23 \text{ mg P g}^{-1} \text{ DM h}^{-1}$  reported by Hao and Chang (1987) for an *Acinetobacter* isolate. The *Acinetobacter* pure cultures studied by Appeldoorn et al. (1992a) released less phosphate ( $1 \text{ mg P g}^{-1} \text{ DM h}^{-1}$  at the most) than phosphate-accumulating sludge (more

than  $1 \text{ mg P g}^{-1} \text{ DM h}^{-1}$  in a full-scale treatment plant and about  $5 \text{ mg P g}^{-1} \text{ DM h}^{-1}$  in a laboratory experiment). Hashimoto and Furukawa (1984) obtained release rates of up to  $0.6 \text{ mg P g}^{-1} \text{ DM h}^{-1}$  for sludge fed with synthetic wastewater. The uptake rates in the studies referred to are given per dry mass of sludge (MLSS), but they have been converted here to dry microbial mass (DM) by assuming that the ratio of DM to MLSS is 0.5 (the ignition loss of the sludge is approximately 50 %).

The better capacity of sludge to release phosphate as compared with *Acinetobacter* pure cultures can also be seen in the amounts of phosphorus released in relation to the amounts bound (%). The initial phosphate concentration in the aerobic experiment is the maximum amount that can be released, assuming that the cells do not contain polyphosphates at the beginning of the experiment. This was the case in the short-term batch experiments. The amounts released by most of the *Acinetobacter* isolates (maximum of 70 % in 24 h) were small as compared with amounts reported in the literature for phosphate accumulating sludge (80 – 90 % in 24 h; Appeldoorn et al. 1992b). However, they were comparable to the amounts released by *Acinetobacter lwoffii* (24 % in 20 h; Fuhs and Chen 1975) and *Acinetobacter* 210A (10 – 20 % in 24 h; Deinema et al. 1985).

According to Deinema et al. (1985) the higher phosphate release rates of sludge as compared with pure cultures may be due to the activated sludge mixed liquor containing also other carbon compounds than acetate, which is frequently used in pure culture studies. These authors found higher release rates for *Acinetobacter* 210A if ethanol or other reduced carbon sources were added together with acetate. Rensink et al. (1985) also observed the same phenomenon with sludge.

Deinema et al. (1985) suggest that actively metabolizing cells release more phosphate than cells in the stationary phase. This would explain the present results obtained after exposing to anaerobic conditions the cultures grown aerobically for 20 to 24 hours. The cultures in the stationary phase when anaerobiosis was introduced, released 8 – 27 % of the phosphate taken up. *A. junii*, isolate 920121, which was not yet in the stationary phase at the beginning of the anaerobic period (c.f. Fig. 14a – d), released 55 – 70 %.

If the hypothesis of Deinema et al. (1985) is correct, the high release percentages obtained for *A. junii*, 920121, in the anaerobic experiments performed after the short aerobic phase with negligible growth (Table 16) indicate that the biomass was just entering the growth phase when anaerobiosis was introduced. A shift to the growth phase after all phosphate has been taken up from the medium is, indeed, likely to take place after excess phosphate uptake through the overplus phenomenon. However, the question remains why the other three *Acinetobacter* isolates treated in the same way as *A. junii*, 920121, did not release more than 27 % of the accumulated phosphorus. On the basis of the present study it seems that different isolates simply have different release rates, and the growth phase has a minor effect.

The effect of different initial phosphate concentrations in the aerobic experiment, i.e. the effect of the amount of polyphosphates stored in the cell, was not specifically studied. In one set of experiments, i.e. the short-term batch culture of *A. junii*, isolate 920121, the results with different initial phosphate concentrations (9 and 17 mg l<sup>-1</sup> P, at pH 6.0) can be compared. The difference in the corresponding release rates (0.22 and 0.26 mg P g<sup>-1</sup> DM h<sup>-1</sup>) is probably not significant. It would be of interest to study whether the release rates as a function of the initial phosphorus content of the biomass follow the Michaelis-Menten or some other model, or if the rates are independent of the phosphorus content. The phosphorus content of the biomass should correlate with the initial concentration of phosphate in the aerobic experiment if the biomasses are constant.

Fairly similar release rates were obtained in the anaerobic experiments conducted after the short-term aerobic incubation of the bacteria, with all phosphate taken up as polyphosphates, and after the bacteria had grown for 20 to 24 hours and part of phosphate had been used for biomass growth. These results seem to indicate that the phosphate release rates are independent of the polyphosphate content of the biomass. The percentages of phosphate released depend, of course, on the polyphosphate content of the biomass.

The mechanisms regulating orthophosphate release in microbes and the factors affecting this reaction are not well known. Natural planktonic

algae and bacteria excrete phosphate under aerobic conditions (e.g. Jansson 1993), while poly-P bacteria are generally thought to require anaerobic conditions (chapter 1.4.2). Whether there is similarity in the phosphate release mechanisms of poly-P bacteria and planktonic microorganisms is not known. For poly-P bacteria it is assumed that the degradation of the low molecule-weight polyphosphates generates energy and the high molecule-weight polyphosphates function as a phosphorus source for the bacteria (Mino et al. 1985, van Groenestijn et al. 1987).

The results of Fuhs and Chen (1975) and Appeldoorn et al. (1992a) suggest that the phosphate released would originate from the low molecule-weight (acid-insoluble) polyphosphates, and the high molecule-weight (acid-soluble) polyphosphates would not be released under anaerobiosis. The *Acinetobacter* pure cultures studied by Appeldoorn et al. (1992a) possessed smaller quantities of low molecule-weight polyphosphates and released less phosphate than sludge. Hiraishi et al. (1989b) found for sludge that low molecule-weight polyphosphates were mainly responsible for the phosphate release, but some of the high molecule-weight fraction was degraded as well. The chain length of polyphosphates may be affected by the specific growth rate, as has been observed for *Candida utilis* by Núñez and Calleri (1989). At high growth rates phosphorus was stored only as low molecule-weight polyphosphates.

The different behaviour of the polyphosphate fractions would explain at least in part the observation in the present study that there could be polyphosphate granules left in *Acinetobacter* cells at the end of the anaerobic period in spite of phosphate release. Lötter (1985) also found granules after anaerobic incubation of an *Acinetobacter* isolate. These granules were probably high molecule-weight polyphosphates which could be utilized as a phosphorus source for growth under aerobic conditions (Mino et al. 1985).

Another explanation for the presence of polyphosphate granules after anaerobiosis is that phosphate release would have continued if the experiment had not been terminated. Beyond a certain stage the Neisser stain probably cannot differentiate between different degrees of polyphosphate storage, and the preparations look the same although the cells may contain variable

amounts of polyphosphates. After the short-term batch experiments all phosphate had apparently been stored as polyphosphates, and there were large amounts of polyphosphates left in the cells after 24 hours of anaerobiosis. In the growing cells only part of the phosphate taken up was stored as polyphosphates, and these cells could release most of their polyphosphates during the anaerobic incubation.

The effect of the various polyphosphate types on phosphate release, as well as the effect of the growth rate on the polyphosphate types, would be important subjects for further studies. If there is a positive correlation between the phosphorus removal capacity and phosphate release, as suggested e.g. by Abu-gharah and Randall (1991), conditions resulting in the formation of polyphosphates that can be released under anaerobic conditions should be favoured. On the other hand, biomass containing polyphosphates not easily released would be easier to handle in the sludge thickener (removes water from sludge after the treatment process; anaerobic conditions may prevail), since phosphate leakage into the effluent would not take place.

Fuhs and Chen (1975) and Ye et al. (1988) found that anaerobic conditions alone did not trigger phosphate release in *Acinetobacter*, but carbon had to be added and pH lowered to 6.0. Adjustment of pH to 6.0 has been found to increase the release rate in sludge as well (Hashimoto and Furukawa 1984). In the present study *Acinetobacter baumannii*, isolate 910045, did not release any phosphate under anaerobic conditions at pH 7.0, although acetate was added (Table 16, Fig. 15a, chapter 3.6). Adjusting pH to 6.0 triggered phosphate release, but the rate remained lower than in the other isolates. Acidic conditions were not, however, a prerequisite for phosphate release for all isolates. *A. junii*, 920121, released more than half of the phosphate bound at both pH values. No significant differences in phosphate release at different pH values could be observed.

Phosphate release in the acinetobacters studied did not always start immediately after introduction of anaerobic conditions. The results indicate that phosphate release may rather be regulated by the phosphate concentration in the medium than the redox potential. Some release was observed under aerobic conditions after phosphate had been exhausted in the growth

medium, and phosphate uptake continued under anaerobic conditions if there was phosphate left in the medium. Similar observations have been made in other studies. Hiraishi et al. (1989b) found that phosphate concentrations above 19 mg l<sup>-1</sup> P inhibited phosphate release in sludge. Fuhs and Chen (1975) observed for *A. lwoffii* that release continued for approximately one hour after the switch from anaerobic to aerobic conditions. Gerber et al. (1987a, b) found that phosphate was released from sludge under aerobic conditions if acetate or propionate was added.

Findings of this kind call for experiments specifically designed to study the dependence of uptake and release on the phosphate concentration in the medium. Furthermore, it would be interesting to study whether a phosphorus-deficient biomass takes up phosphate under originally anaerobic conditions, or whether release and uptake occur simultaneously, as reported by Gerber et al. (1987a) for sludge. Studies on the kinetics of the enzymes involved in polyphosphate metabolism would clarify this question.

Both inhibition of phosphate release under anaerobic conditions and phosphate release taking place under aerobic conditions are undesirable phenomena. The former may result in a low phosphate uptake rate during the aerobic period and the latter in leakage of phosphate in the wrong phase of the treatment process.

Already Fuhs and Chen (1975) have suggested that the correlation between anaerobiosis and phosphate release is indirect. Anaerobic conditions may enhance biological phosphorus removal by producing through fermentation volatile fatty acids, which are suitable carbon sources for acinetobacters, and carbon dioxide, which lowers the pH. Anaerobiosis, when nitrate is not present, also results in selection of acinetobacters against other strict aerobes and denitrifiers competing for volatile fatty acids.

#### 4.5 Denitrification potential of *Acinetobacter* isolates

The denitrification ability of acinetobacters is yet another interesting question in biological nutrient removal. If acinetobacters were able to denitrify, the same biomass would be able to

remove both phosphorus and nitrogen. This ability would have practical implications in the design of treatment plants.

Acinetobacters are usually considered to be unable to denitrify (Juni 1984). Nevertheless Focht and Joseph (1974), Lötter (1985) and Lötter et al. (1986) have reported on acinetobacters that were able to use nitrate as an electron acceptor when oxygen was not present. These acinetobacters were isolated in sewage or activated sludge treating municipal wastewater. The proportion of nitrate reduced to gas is not given in these reports, and it is not clear whether the criteria set for respiratory denitrification, i.e. 80 % of the nitrate-nitrogen reduced to gas (Tiedje 1988), were met. Lötter (1985) measured only nitrite, but not gaseous nitrogen formation, and so it is uncertain if nitrite was further reduced to gas. The validity of the identification of these bacteria as acinetobacters is also questionable. Focht and Joseph (1974) performed a set of traditional biochemical tests, and Lötter (1985) and Lötter et al. (1986) used a fluorescent antibody technique followed by the API 20E identification system designed for enteric bacteria (in the present study API 20NE for non-enteric bacteria was used).

Kuba et al. (1993) found that denitrifying sludge accumulated polyphosphate. Bacteria were not identified, but cycles of phosphate uptake and release similar to those in sludge enriched with acinetobacters were observed. The denitrifying bacteria in this sludge could not, however, be acinetobacters, since the process did not include an aerobic phase.

The results of the present study support the general view that acinetobacters are unable to denitrify. It may, however, be argued that activated sludge treating forest industry wastewaters is an unlikely environment where to find denitrifying acinetobacters. In the treatment plants of pulp and paper mills both the nitrate concentrations and the nitrification activities (conversion of ammonium to nitrate) are usually low as compared with the municipal wastewater treatment plants (Jørgensen and Pauli 1992). However, denitrification potential was not found even in acinetobacters isolated from a pilot plant treating municipal wastewater (Jørgensen and Pauli, manuscript in preparation).

## 5. CONCLUSIONS

### Acinetobacters

Enhanced biological phosphorus removal is based on the ability of some bacteria (poly-P bacteria) to accumulate excess phosphorus in their cells. Acinetobacters are likely to be among the most significant poly-P bacteria in activated sludge with enhanced phosphorus removal.

According to the present study there were acinetobacters in sludge treating wastewaters of pulp and paper production, although their numbers were low. In some samples of influent wastewater acinetobacters were among the most common cultivable bacteria. Acinetobacters were not, however, enriched in the sludge, where they were only occasionally found among the most dominant bacteria.

Acinetobacters could be easily enriched from sludge under aerobic conditions in liquid acetate mineral medium. The results indicate that enhanced biological phosphorus removal may be achieved in forest industry treatment plants if the growth of aerobes other than acinetobacters can be limited (and carbon limitations overcome).

In practice, introducing alternating aerobic and anaerobic sequences would be a means of favouring acinetobacters over other strictly aerobic bacteria unable to form intracellular polyphosphate and carbon storages.

Most of the bacteria isolated on the acetate mineral medium used to select for acinetobacters were not acinetobacters. It is obvious that the number of colony forming units on this medium cannot be used as a presumptive number of acinetobacters. Among the isolates *Pseudomonas* sp. was the most common of the identifiable bacteria. Many isolates could not be identified with the API 20NE identification kit for non-enteric bacteria. Improved identification systems for sludge bacteria are therefore needed.

### Optimal growth conditions

On the basis of this study and information provided by the literature, lack of suitable carbon compounds (volatile fatty acids) is an important factor limiting the growth and enrichment of



acinetobacters in the sludge treating wastewaters of sulphate pulp mills. This problem could be overcome for instance by using special anaerobic reactors for generating fermentation products from sludge and wastewater.

According to the present study wastewater from integrates producing mechanical pulp and paper may contain significant amounts of volatile fatty acids. In this case enhanced biological phosphate removal might be feasible even without a fermentative reactor if the treatment process favoured enrichment of acinetobacters.

There seemed to be enough of other elements (K, Mg, Ca) essential for maximum growth and polyphosphate metabolism of acinetobacters in the treatment plants studied.

The growth of the *Acinetobacter* isolates studied was enhanced with a higher nitrogen to phosphorus ratio in the medium than is common in the forest industry wastewaters. However, although a high growth rate is important for biomass production and competition with other bacteria, it does not necessarily result in maximal polyphosphate accumulation. According to the literature the relationship between polyphosphate accumulation and growth is variable.

### Phosphate uptake

Phosphate-starved acinetobacters, as well as many other bacteria, are able to take up phosphate immediately after being exposed to phosphate (the overplus phenomenon). The phosphate uptake rates of other bacteria capable of this kind of excess uptake may be lower than the rates of acinetobacters, as shown in this study for *Agrobacter radiobacter*, or higher, which could not be shown in the present study with the phosphate uptake medium used.

Acinetobacters are among the few bacteria known to take up excess phosphate during the growth phase under favourable growth conditions as well (luxury uptake). In wastewaters with low orthophosphate concentrations, most of the orthophosphate is likely to be taken up through the overplus phenomenon immediately after the wastewater is mixed with the recycled sludge. Luxury uptake by growing bacteria may be important in the assimilation of phosphate

generated by the hydrolysis of organic phosphorus compounds later in the treatment process.

The phosphate uptake of the *Acinetobacter* isolates followed the Michaelis-Menten (or Monod) model without significant deviations in either type of excess uptake (overplus and luxury). One of the isolates continued to grow at the expense of the internal polyphosphate storage after exhaustion of phosphate in the medium. The Michaelis-Menten model cannot be used to describe this phenomenon.

The kinetic parameters obtained for the *Acinetobacter* isolates varied. One of the isolates, which originated from influent wastewater with a low phosphate concentration, had a much more efficient uptake system for phosphate than the other isolates originating from enrichment cultures or sludge with added phosphorus.

According to this study, the *Acinetobacter* populations present in the aerobic treatment processes may remove only a few percent of the phosphate in the influent wastewater. The relatively high phosphate uptake rates cannot compensate for the low numbers of these bacteria in the sludge.

### Phosphate release

Phosphate release, which is considered to be a prerequisite for efficient phosphorus removal by activated sludge, was different in the *Acinetobacter* isolates studied. Only one of the isolates released phosphate in amounts comparable to the amounts reported for sludge with enhanced phosphorus removal. This isolate was also the most efficient in phosphate uptake.

The results indicate that the redox potential is probably not crucial for polyphosphate degradation (i.e. phosphate release) and formation (i.e. phosphate uptake). Instead, these reactions may be determined by other factors, such as pH and phosphate concentration in the medium. It may be also hypothesized that phosphate release starts only after the exhaustion of phosphate in the growth medium and, *vice versa*, phosphate uptake continues as long as there is phosphate in the medium. Both reactions would seem to take place irrespective of the redox potential.

The findings of this study support the theory

presented in the literature that the anaerobic phase where no nitrate is present is needed in enhanced biological phosphorus removal mainly for production of suitable carbon sources and for selection of acinetobacters (and probably other poly-P bacteria) against other strictly aerobic bacteria and denitrifiers in activated sludge.

### Denitrification potential

Acinetobacters isolated in wastewater and activated sludge did not denitrify. This finding is in accordance with the common view of the properties of acinetobacters, but contradicts some results presented in the literature that acinetobacters in municipal sludge may have denitrification abilities. It is not, however, certain if the criterium set for true denitrification was met in those studies. The validity of identification of these bacteria as acinetobacters can also be questioned.

### Monitoring of acinetobacters

Because there may be significant differences in the phosphate uptake and release kinetics of acinetobacters, monitoring of *Acinetobacter* numbers alone does not produce enough information on the phosphorus removal potential of sludge. In order to obtain reliable estimates of *Acinetobacter* numbers by cultivation laborous identification of the bacteria would also be required, since many of the colonies growing on the medium used as a selective medium for acinetobacters may actually be other bacteria. A more practicable method for monitoring purposes might be for instance to determine the activities of the enzymes involved in polyphosphate metabolism, as presented in the literature.

### Biological phosphorus removal as an alternative to conventional activated sludge treatment

Nowadays some of the existing aerobic activated sludge plants of pulp and paper mills can be operated successfully without phosphorus additions. If this could be done in all existing plants there would be no reason to modify their

processes for enhanced biological phosphorus removal, although in some cases a modification might be possible with only minor changes.

However, when new treatment plants are being designed and built, biological phosphate removal would be worth considering as an alternative to the conventional aerobic activated sludge processes in forest industry as well. In new plants the requirements of acinetobacters and other poly-P bacteria could be taken into account from the very beginning.

### Other applications concerning acinetobacters

The findings of this study may be useful, at least to some extent, in the treatment of municipal and other types of wastewater as well, in studies of the degradation of hydrocarbons and other recalcitrant compounds, as well as in studies concerning eutrophication and exchange of phosphorus between sediment and water.

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Anneli Pauli

## YHTEENVETO

Metsäteollisuuden fosforikuormitukseen on viime vuosina kiinnitetty huomiota sisä- ja rannikkovesien lisääntyvän rehevöitymisen myötä. Vaikka näiden jätevesien fosforipitoisuudet ovat suhteellisen alhaisia, suurien vesimäärien vuoksi fosforikuormitus saattaa olla merkittävä.

Biologinen fosforinpoisto, joka perustuu eräiden bakteerien kykyyn varastoida ylimääräistä fosforia polyfosfaatteina soluihinsa, on vaihtoehto perinteiselle aerobiselle aktiivilieteprosessille.

Merkittävimpiä fosforin varastoijina pidetään yleensä aerobisia, *Acinetobacter*-sukuun kuuluvia bakteereita, jotka erikoislaatuisen fosfori- ja hiili-metaboliensa ansiosta tulevat tilapäisesti toimeen myös anaerobisissa olosuhteissa. Tässä tutkimuksessa polyfosfaatteja varastoivien bakteerien malliorganismeina käytettiin puhdistamolle tulevas- ta jätevedestä sekä aktiivilietteestä ja lietteiden rikastusviljelmistä eristettyjä *Acinetobacter*-kantoja.

Tässä työssä tutkittiin: (1) *Acinetobacterien* pitoisuuksia sulfaattisellu- ja paperitehtaiden jätevesissä ja aktiivilietteissä, (2) *Acinetobacterien* kasvulle ja polyfosfaattimetabolialle tärkeiden haihtuvien rasvahappojen sekä kaliumin ja magnesiumin optimaalisia pitoisuuksia sekä optimaalisia typpi-fosforisuhteita kasvualustassa, (3) Fosfaatin sitoutumis- ja vapautumisnopeuksia aerobisissa ja anaerobisissa olosuhteissa, (4) *Acinetobacterien* merkitystä nykyisten aerobisten aktiivilietelaitosten fosforinpoistossa ja (5) *Acinetobacterien* denitrifikaatiokykyä.

## Acinetobakteerit

Puhdistamolle tulevan jäteveden sekä aktiivilietteen bakteeristoa tutkittiin kahden sulfaattisellutehtaan ja kolmen paperitehtaan puhdistamolla vuosina 1991 – 1992. *Acinetobacterien* osuus oli yleensä alle 2 % tryptoni-glukoosi-hiiva-alustalla (yleisalusta) kasvaneiden aerobisten, heterotrofisten bakteerien kokonaismäärästä. *Acinetobakteereita* oli kuitenkin helppo rikastaa asetaatti-mineraaliliuoksessa sekä sellu- että paperitehtaiden lietteistä. Tämä viittaa siihen, että *acinetobacterien* toimintaan perustuva biologinen fosforinpoisto voi sopivissa olosuhteissa käynnistyä helpostikin, vaikka *acinetobakteerit* eivät alun perin kuuluisikaan lietteen yleisimpiin bakteereihin. Puhdistamoissa *acinetobacterien* rikastumista voidaan edistää järjestämällä prosessiin anaerobinen vaihe, joka rajoittaa muiden aerobisten bakteerien kasvua.

Useimmat *acinetobacterien* selektoimiseen yleisesti käytetyllä asetaatti-mineraalialustalla kasvaneista bakteereista eivät olleet *acinetobakteereita*. Yleisin tunnistettu bakteeri oli *Pseudomonas* sp. Huonon selektointikykyänsä vuoksi tällä alustalla kasvavien bakteerien määrää ei voida käyttää edes *acinetobakteripitoisuuksien* karkeaan arviointiin. API 20NE – identifiointisytee-

millä ei voitu tunnistaa läheskään kaikkia lietteen bakteereita.

## Optimaaliset kasvuolosuhteet

*Acinetobacterien* optimaalisia kasvuolosuhteita hiilen (asetaattina), typen, fosforin, kaliumin ja magnesiumin suhteen tutkittiin puhdasviljelmillä faktorikokein.

*Acinetobakteereille* parhaita hiilen- ja energianlähteitä ovat lyhytketjuiset, haihtuvat rasvahapot. Tutkitulta sulfaattisellutehtaalta aktiivilietelaitokselle tulevassa jätevedessä haihtuvien rasvahappojen pitoisuudet olivat hyvin alhaisia. On todennäköistä, että sopivien hiiliyhdisteiden puute on merkittävä *acinetobacterien* kasvua rajoittava tekijä sellutehtaiden jätevesissä. Jatko-tutkimuksissa tulisi selvittää, miten helposti rasvahappoja voitaisiin tuottaa fermentaation avulla jäteveden ja lietteen suurimolekyylisistä yhdisteistä.

Paperitehtaiden jätevesissä haihtuvia rasvahappoja (eniten etikkahappoa) oli runsaasti. Näissä jätevesissä biologinen fosforinpoisto saattaisi helpostikin käynnistyä, jos olosuhteet muuten suosisivat *acinetobakteereita*.

Tutkituilta sellu- ja paperitehtailta tulevissa jätevesissä oli *acinetobacterien* optimaaliseen kasvuun riittävät määrät kaliumia ja magnesiumia.

*Acinetobakteerit* kasvoivat parhaiten alustassa, jonka typpi-fosforisuhde oli noin 10. Tämä on kaksinkertainen verrattuna jätevesien puhdistuksessa optimaalisena pidettyyn suhteeseen. Tulos viittaa siihen, että *acinetobacterien* kasvua voitaisiin suosia korkeammilla typpi-fosforisuhteilla kuin puhdistusprosesseissa on tavanomaista.

Suuri kasvunopeus on tärkeä biomassan muodostuksessa ja kilpailussa muiden bakteerien kanssa. Fosfaatin akkumulaatio ei kuitenkaan välttämättä ole maksimaalista samoissa olosuhteissa kuin kasvu. Kirjallisuudessa esiintyy vaihtelevia tietoja kasvun ja fosfaatin akkumulaation välisestä suhteesta.

## Fosfaatinotto

Vähäfosfaattisesta kasvuympäristöstä fosfaattia sisältävään ympäristöön siirretyt *acinetobakteerit* assimiloivat nopeasti fosforin ja varastoivat sen polyfosfaatteina (ns. overplus-ilmiö). Tämän

tyyppiseen ylimääräisen fosforin varastoitumiseen kykenevät monet muutkin bakteerit, mutta niiden fosfaatinottonopeudet saattavat ovat alhaisempia kuin acinetobakteereilla, kuten tässä tutkimuksessa todettiin *Agrobacter radiobacter*ille. Huomattava osa puhdistamolle tulevan jäteveden fosfaatista voi sitoutua bakteereihin overplus-ilmion kautta jo puhdistusprosessin alussa, kun palautusliete sekoitetaan jäteveden kanssa.

Acinetobakteerit voivat, toisin kuin useimmat muut bakteerit, varastoida ylimääräistä fosforia myös kasvuvaiheen aikana hyvissä kasvuolosuhteissa (ns. luxury uptake). Tämän tyyppinen fosfaatinotto saattaa olla tärkeää orgaanisten fosforiyhdisteiden hydrolyysissä vapautuvan fosfaatin assimiloinnissa.

*Acinetobacter*-puhdasviljelmien fosfaatinotto asetaatti-mineraaliliuoksessa noudatti Michaelis-Mententin kineettistä mallia sekä overplus-ilmiota jäljittelevissä kokeissa että optimaalisissa kasvuolosuhteissa. Acinetobakteerien kasvua ja fosfaatinottoa kuvaavat kineettiset parametrit vaihtelivat. Tehokkainta fosfaatinottoa oli kannalla, joka oli eristetty vähäfosforisesta tulevasta jätevedestä. Puhdistamolla fosforilisäyksiä saaneesta lietteestä eristetyn kannan sekä rikastusviljelmistä eristettyjen kantojen fosfaatinottotehokkuuksissa ei ollut eroja. Tehokkain kanta kasvoi varastoimansa polyfosfaatin varassa sen jälkeen, kun alustan fosfaatti oli loppunut.

Metsäteollisuuden jätevesissä esiintyvillä fosfaattipitoisuuksilla tietyn *Acinetobacter*-kannan fosfaatinottonopeus oli lähes samanlaista sekä overplus-ilmiota että luxury uptake-tapahtumaa kuvaavien kineettisten mallien avulla laskettuna. Malleilla laskettujen ottonopeuksien erot kasvativat fosfaattipitoisuuksien kasvaessa.

Acinetobakteerien fosfaatinottonopeus oli suhteellisen suuri. Se ei kuitenkaan voinut kompensoida näiden bakteerien pientä määrää nykyisissä aerobisissa aktiivilietepuhdistamoissa, joissa acinetobakteerien voidaan arvioida poistavan enintään muutaman prosentin jäteveden fosforikuormasta.

### Fosfaatin vapautuminen

Myös fosfaatin vapautuminen, joka on tarpeen tiettyssä fosforinpoistoprosessin vaiheessa, vaihteli *Acinetobacter*-kantojen välillä. Vain se kanta, joka oli tehokkain fosfaatinotossa, vapautti anaerobi-

sisä oloissa fosfaattia yhtä suuria määriä kuin mitä on raportoitu tehokkaasti fosforia poistavien lietteiden vapauttavan. Fosfaatin vapautuminen näytti riippuvan enemmän kasvualustan pH-arvosta sekä fosfaattipitoisuudesta kuin redox-potentiaalista. Kokeet viittaavat siihen, että fosfaatin vapautuminen alkaa vasta, kun kasvualustan fosfaatti on loppunut, ja että toisaalta fosfaatinotto jatkuu niin kauan kuin fosfaattia on käytettävissä, redox-potentiaalista riippumatta.

Havainnot tukevat kirjallisuudessa esiintyvää teoriaa, jonka mukaan anaerobinen vaihe ei ole välttämätön fosfaatin vapautumiselle. Teorian mukaan anaerobiaa tarvitaan sopivien hiiliyhdisteiden tuottamiseksi fermentaation avulla, sekä acinetobakteerien rikastamiseksi monien muiden aerobisten bakteerien eliminoituessa.

### Denitrifikaatiopotentiaali

Acinetobakteerien mahdollinen denitrifikaatio-kyky on kiinnostava kysymys pohdittaessa fosforin ja typen samanaikaista poistoa. Yksikään tutkituista 57 *Acinetobacter*-kannasta ei pelkistänyt nitraattia kaasuksi, eli ei denitrifioinut. Tulos on sopusoinnussa useimpien kirjallisuustietojen kanssa.

### Acinetobakteerien monitorointi

Koska jo muutamalla *Acinetobacter*-kannalla tehty kokeet osoittivat, että fosfaatin otto ja vapautuminen vaihtelevat selvästi kantojen välillä, ei lietteen fosforinpoistokykyä pystytä arvioimaan, vaikka acinetobakteerien määrä lietteessä tiedettäisiinkin. Lisäksi acinetobakteeripitoisuuksien määrittäminen edellyttäisi työlästä bakteeripäkkeiden tunnistamista, koska ei ole olemassa riittävän selektiivistä kasvualustaa. Jatkotutkimuksissa voisi kiinnittää huomiota pikemminkin polyfosfaattimetaboliaan osallistuvien entsyymien aktiivisuuksien määrittämiseen arvioitaessa lietteen fosforinpoisto-ominaisuuksia.

### Biologinen fosforinpoisto vaihtoehtona metsäteollisuuden jätevesien käsittelyssä

Tämän tutkimuksen tulokset viittaavat siihen, että biologinen fosforinpoisto on varteenotettava

vaihtoehto metsäteollisuuden jätevesien käsittelyssä. Edellytyksenä on kuitenkin anaerobisen vaiheen järjestäminen puhdistusprosessiin. Sulfaattisellutehtaiden jätevesien käsittelyssä biologisen fosforinpoiston aikaansaaminen edellyttäisi lisäksi suurimolekyylisten yhdisteiden fermentointia.

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